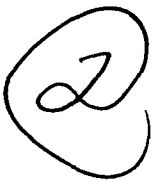

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A purified diet has been developed and proven adequate for growth of the medaka (Oryzias latipes), a sensitive teleost species proven responsive to hepatocarcinogenic agents. The overall nutritional adequacy of the diet, a casein based ration, was evaluated and compared to three diets: commercially available flaked fish food; live newly hatched Artemia; and a combination of flake diet supplemented with Artemia. Survival, growth, reproductive success, general and liver histopathology, and selected hepatic enzyme activities were compared in medaka from first feeding through reproductive maturity. The PC-diet proved adequate in all of the above criteria. This diet provides a standardized, nutritionally adequate and consistent alternative to undefined conventional diets and is less likely to contain the range of xenobiotics possible in whole live food. Studies in progress indicate the suitability of the diet for aquatic toxicity/carcinogenicity studies.

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David E. Hinton 12-13-91

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INTRODUCTION

The medaka (*Oryzias latipes*) has been used extensively to investigate experimental embryology in biology laboratories (Kirchen and West 1976; Yamamoto and Egami 1974). Within the last two decades, work performed by Japanese investigators (Aoki and Matsudaira 1977; Aoki and Matsudaira 1981; Aoki and Matsudaira 1984; Aoki and Matsudaira 1986; Egami and Etoh 1969; Egami, Kyono-Hamaguchi, et al. 1981; Ishikawa, Masakito, et al. 1984; Ishikawa, Shimamine, et al. 1975; Ishikawa and Takayama 1979; Kyono 1978; Kyono and Egami 1977; Kyono, Shima, et al. 1979; Kyono-Hamaguchi 1984; Masahito, Aoki, et al. 1989; Masahito, Ishikawa, et al. 1988; Matsushima and Sugimura 1976; Takayama and Ishikawa 1977) and subsequently by U.S. investigators (Hawkins, Fournie, et al. 1991; Hawkins, Fournie, et al. 1986; Hawkins, Overstreet, et al. 1985a; Hawkins, Overstreet, et al. 1988a; Hawkins, Overstreet, et al. 1988b; Hawkins, Overstreet, et al. 1985b; Hawkins, Walker, et al. 1990; Hawkins, Walker, et al. 1988c; Hinton 1989a; Hinton, In Press a; Hinton, Couch, et al. 1988b; Hinton, Hampton, et al. 1985; Hinton, Lantz, et al. 1984a; Hinton, Laurén, et al. 1988a; Hinton, Teh, et al., In Press; Klaunig, Barut, et al. 1984; Laurén, Teh, et al. 1990) have shown that this fish species has great promise as a vertebrate model for toxicity including carcinogenicity. The time to tumor when the animal is exposed to diethylnitrosamine or to methylazoxymethanol acetate is brief. The latency period for hepatocarcinogenesis with these compounds is frequently 8 - 16 weeks. In addition to the response to carcinogens in the laboratory, this fish with its small size, ease of rearing, and other economic advantages (Laurén, Teh, et al. 1990; Matsushima and Sugimura 1976) is being used to investigate toxicity and carcinogenicity of extracts from sediments at various polluted harbors and estuaries (Fabacher, Besser, et al. 1991).

Although the Japanese medaka (*Oryzias latipes*) shows great promise as a model for

carcinogenesis (Aoki and Matsudaira 1977; Egami, Kyono-Hamaguchi, et al. 1981; Ishikawa, Shimamine, et al. 1975; Ishikawa and Takayama 1979), its potential is currently limited by the lack of a defined diet. Refinements in detection of early preneoplastic lesions and improved definition of windows of bioavailability consistent with sensitivity in developing host are needed.

Current, closed-formula, commercial rations are derived largely from fish meal and plankton of unknown source and variability and may contribute confounding environmental contaminants. Since these diets are flaked, attempts to add test compounds for the purpose of conducting chronic dietary exposures have been made more difficult. The result is that we are limited to studies with so-called complete carcinogens (Aoki and Matsudaira 1977; Egami, Kyono-Hamaguchi, et al. 1981; Ishikawa, Shimamine, et al. 1975; Ishikawa and Takayama 1979). It is generally agreed that the number of environmentally relevant chemicals which modulate (promote or inhibit) carcinogenesis exceeds the number of initiators of the process. Studies of modulation of carcinogenesis require a model which consistently provides an expected tumor incidence with a given dose of initiating carcinogen. Furthermore, since dietary contaminants are important modulators of carcinogenesis, a highly purified, defined diet to which test agents may be added is essential.

We seek to develop an open-formula defined diet (OFD) for use in carcinogenesis studies with medaka. Advantages of using medaka as *in vivo* screens in cancer bioassay have been reviewed (Consensus Committee 1984; Hatanaka, Doke, et al. 1982; Matsushima and Sugimura 1976). Although a partially defined diet has been available (Sinnhuber, Hendricks, et al. 1977) and used to investigate modulation of carcinogenesis in the rainbow rainbow trout (*Oncorhynchus mykiss*) model (Nixon 1984), environmental characteristics of medaka require use of a diet more

suitable for warm water species. In contrast to the larger trout, amount of total diet required for medaka makes use of a defined ration cost effective.

The NCI and US EPA Consensus Committee (Consensus Committee 1984) recommended use of defined rations so that attempts to analyze modulatory effects of environmentally relevant chemicals on carcinogenesis in this promising model would be possible. The UC Davis Aquatic Toxicology Laboratory with expertise in anatomy, pathology, physiology, biochemistry, toxicology and nutrition of fishes, has undertaken research to refine elements of this promising vertebrate model for carcinogenesis.

The development of new, faster eukaryotic models for bioassay to identify potential carcinogens and modulators of carcinogenesis is a pressing need for research. As is stated above, medaka develop tumors of liver after exposure to proven mammalian kenotoxic carcinogens. Their brief latency periods, sensitivity to a variety of procarcinogens, and decreased cost while maintaining large number of exposed individuals argue for further development of this model. However, the lack of a defined diet and absence of established tumor incidence data under standardized and reproducible conditions are current limitations for development. The overall goal of the research about which this midterm report is addressed, is to develop a sensitive fish model for studying carcinogenic potential and modulatory effects of environmental agent on carcinogenesis. To reach this goal, we have identified four enabling objectives: 1) produce a nutritionally adequate, open-formula purified diet for medaka; 2) determine levels of xenobiotic metabolizing enzymes in medaka fed this and a conventional diet; 3) establish incidences of liver tumors in medaka fed the open-formula diet; and 4) compare the frequency of liver tumor formation in carcinogen-initiated medaka fed the open-formula diet versus those fed a

conventional dietary regime. During the two years of this work, we have addressed the first and third objectives as listed above and have made partial progress with regard to objectives numbers 2 and 4. What follows is a full report on a nutritionally adequate, open-formula purified diet for medaka and the growth and overall health characteristics of fish fed this versus a conventional regime (DeKoven, et al. 1991) (Chapter 1). In Chapter 2, we shall present our results from an ongoing study comparing frequency of liver tumor formation in medaka exposed to an identical carcinogen and fed either the open-formula purified ration or a conventional dietary regimen.

Chapter 1

Purified Diet for medaka (*Oryzias latipes*): Refining a Fish Model for Toxicological Research

Introduction:

Small aquarium fishes are receiving increasing attention as vertebrate test organisms for rapid *in vivo* screening of suspect carcinogens and as monitors of environmental pollution in aquatic field surveys (Hoover 1984a; Vogelbein, Fournie, et al. 1990). One of the most promising small fish species is the medaka orange-red or golden variety) (Aoki and Matsudaira 1977; Bunton 1990; Egami, Kyono-Hamaguchi, et al. 1981; Hinton, Couch, et al. 1988b; Hinton, Lantz, et al. 1984a; Kyono and Egami 1977; Laurén, Teh, et al. 1990). Medaka are tolerant to a wide range of environmental conditions, and their small size and rapid maturation supports uncomplicated breeding and maintenance of large numbers in the laboratory. Aspects of the physiology, development and genetics of this species are known (Briggs and Egami 1959; Yamamoto 1975). Medaka have proven to be particularly sensitive to a variety of carcinogens (Hatanaka, Doke, et al. 1982), forming tumors after relatively short (3 - 4 months) induction periods (Hawkins, Overstreet, et al. 1988b; Masahito, Aoki, et al. 1989). In addition, individual medaka exhibit hepatic enzyme responses to polyaromatic hydrocarbon (PAH) xenobiotics similar to other vertebrate models (Schell, Cooper, et al. 1987).

Given the potential importance of medaka as a vertebrate model, the need exists for a nutritionally adequate and consistent experimental diet for this species (Bailey, Hendricks, et al. 1984a; Consensus Committee 1984). Conventional medaka diets, such as *Artemia* nauplii and/or

closed-formula, commercially available flake diets, are not ideal for most carcinogenesis or toxicity bioassays because of the risk of additional contaminant exposure through the diet which may alter the toxic response of the fish (Consensus Committee 1984). Live foods, such as *Artemia* nauplii, can be a source of adventitious xenobiotics (Olney, Schauer, et al. 1980; Seidel, Johns, et al. 1982). Additionally, different geographical strains of *Artemia* cysts may contain varying amounts and types of chlorinated and aromatic hydrocarbons and heavy metals (Olney, Schauer, et al. 1980; Seidel, Johns, et al. 1982). Although adventitious contaminants in non-purified commercial fish feeds have not been evaluated, changes in liver ultrastructure, including accumulation of lipid droplets and proliferation of rough endoplasmic reticulum, have been reported in fish fed non-purified, "practical" feed ingredients common to commercially available diets (Affandi and Biagianti 1987; Bac, Biagianti, et al. 1983; Mosconi-Bac 1987). Additionally, the incidence of liver tumors, after exposure to identical concentrations of aflatoxin B_1, was significantly higher in rainbow trout (*Oncorhynchus mykiss*) fed a diet based on fish protein concentrate compared to trout fed purified casein-based diets (Lee, Sinnhuber, et al. 1978).

While nutritional adequacy of conventional diets for medaka has yet to be rigorously evaluated, decreased growth and reproduction have been noted when commonly used flake feeds were fed alone (Hirshfield 1980; Stanley 1977). Although *Artemia* nauplii generally support good growth and survival, their nutrient composition may vary depending on geographical strain or season during which the cysts were collected (Cowgill, Emmel, et al. 1987; Fujita, Watanabe, et al. 1980; Klein-Macphee, Howell, et al. 1980; Schauer, Johns, et al. 1980; Seidel, Johns, et al. 1982). Such variations significantly affect growth and survival of both fish and crustacean larvae

(Amat, Hontoria, et al. 1987; Beck, Bengston, et al. 1980; Johns, Peters, et al. 1980; Klein-Macphee, Howell, et al. 1980; Klein-Macphee, Huntting Howell, et al. 1982; Seidel, Johns, et al. 1982).

Neither the effect of diet nor the nutritional status of medaka have been considered when evaluating metabolic or tumorigenic responses to experimental toxicants. Several studies with small mammals and other fish species, however, indicate that the nutritional status of the experimental animals and nutritional variation between different experimental diets may influence metabolic response and sensitivity to xenobiotics (Andersson, Koivusaari, et al. 1985b; Ankley and Blazer 1988; Hickie and Dixon 1987; Mehrle, Johnson, et al. 1974; Mehrle, Mayer, et al. 1977; Sachan 1975; Stott and Sinnhuber 1987; Wade, White, et al. 1985).

A standardized nutritionally adequate purified diet, suitable for maintaining medaka through all life stages, would overcome many of the problems associated with conventional diets (Bailey, Hendricks, et al. 1984a). A purified diet would be more nutritionally consistent and less likely to contribute xenobiotics. Purified diets have been successfully developed for rainbow trout and used in toxicity bioassays and long-term feeding studies (Halver 1957; Halver and Coates 1957; Hendricks 1982; Yu, Sinnhuber, et al. 1979); however, these diets were not formulated for warm water species such as the medaka. To date, purified diets have not been used to rear medaka from first feeding through reproductive maturity.

A purified casein-based diet (PC-diet), was formulated (DeKoven 1990) by modifying a purified ration developed by Conklin *et al.* (Conklin, D'Abramo, et al. 1980). Two dietary trials were undertaken to evaluate the nutritional adequacy of PC-diet compared to conventional feeding regimes presently used in other laboratories (Aoki and Matsudaira 1977; Bunton 1990;

Egami, Kyono-Hamaguchi, et al. 1981; Schell, Cooper, et al. 1987). Medaka growth, survival, reproductive success including embryo performance, general histology, and activities of selected hepatic enzymes were evaluated and compared.

Materials & Methods:

Diets: Both formulated and live diets were used. Trial 1 compared the nutritional adequacy of the purified casein-based diet (designated as PC-diet) to a live food (A-diet) and a closed formula, commercially available flaked diet for tropical fish (FL-diet). The A-diet consisted of newly hatched brine shrimp (*Artemia* spp.) nauplii (San Francisco Bay Brand, Newark, CA), while the FL-diet consisted of a conventionally used commercial flake diet, Kordon Stress Flakes (Kordon Co., Hayward, CA). In Trial 2, medaka were fed either the PC-diet or a combined regime of a commercial flake diet (Tetramin, Tetrawerke, Germany[1]) plus brine shrimp nauplii (designated as F/A-diet). The composition of the PC-diet is given in Table 1 and proximate analyses (Jones 1984) of the PC-, FL- and A-diets are shown in Table 2.

The formulated diets were prepared in 100 g (Trial 1) or 1 kg (Trial 2) batches and stored at -20°C. Purified ingredients were obtained from U.S. Biochemical Corporation (Cleveland, OH) and ICN Nutritional Biochemicals (Cleveland, OH). Dry ingredients for the PC-diets were mixed with a rotary mixer for 15 minutes. The oils were mixed with the tert-butyl hydroquinone (TBHQ; Aldrich Chemicals, Milwaukee, Wisconsin) and mixed well into the dry ingredients. Distilled water was slowly added to form a slightly cohesive mixture. In Trial 1, the PC-diet was then pressed through a nylon sieve (1 mm mesh size) and dried at 50°C for 15 minutes. In Trial 2, the diet was pressed through a stainless steel sieve (1.4 mm mesh size, U.S. Standard Tyler Sieve, #14) and freeze dried at - 80°C in a Labconco freeze dryer.

In both trials, fish were fed to slight excess twice daily and tanks were siphoned daily to remove uneaten food and feces. The F/A-diet group was fed flaked food five days per week and brine shrimp nauplii two days per week. In both trials, particle sizes of the formulated diets were increased as the fish grew. From hatch to 4 weeks, the fish were fed formulated diets ranging from 100 to 250 µm particle diameter. After 4 weeks, the fish were fed particle sizes ranging from 250-µm to 850-µm particle diameter. Newly hatched brine shrimp nauplii (A-diet and F/A-diet) were separated from unhatched and empty cysts and rinsed with distilled water before being fed to the fish.

System design: Golden variety medaka were reared in a static system (Trial 1: larvae and juveniles) or in a recirculating aquarium system (Trial 1: adults; Trial 2: all life stages) (Nunez and Hinton In Prep.). Water temperature was maintained at 25 ± 0.5°C. Ammonia and nitrite levels were monitored daily (Trial 1) or weekly (Trial 2) and maintained at $\leq$ 0.1 ppm. Nitrates, pH, conductivity and hardness were monitored weekly in Trial 2. Dissolved O_2 was maintained at or near saturation.

Experimental Methods: Medaka eggs were collected from broodstock maintained at 25°C under a 16L:8D photoperiod. Eggs for all experiments were pooled from several females and incubated in aerated modified (no methylene blue) embryo rearing media (Kirchen and West 1976; Rugh 1962) at 25 ± 1°C. The embryo rearing media was replaced daily at which time dead embryos were removed. Larvae hatched 9 to 10 days after the eggs were collected.

In both trials, initial mean wet and dry weights and morphometric parameters were determined by sampling 30 newly hatched unfed normal larvae. The larvae were killed with an overdose of tricaine methane sulfonate ("Finquel", Argent Co., Redmond WA). Standard lengths

(length from the tip of the snout to the end of the vertebral column) were measured in Trial 1 using an ocular micrometer and a dissecting microscope. Morphometric analyses of total length, maximum depth and maximum width were measured in Trial 2 using a computer-assisted image analysis system (Nikon Micro-plan II, Laboratory Computer Systems Inc., Cambridge, MA) and a dissecting microscope. Euthanized larvae were then carefully wicked dry, placed in pre-weighed foil cups and weighed on an ultramicrobalance to 0.00001 g (Trial 1) or 0.0001 g (Trial 2). The larvae were dried at 60°C for 24 h, cooled in a desiccator and then weighed.

Trial 1

Newly hatched larvae with normal swim bladder inflation were randomly sorted and equally distributed among nine 1.5-L glass jars, 20 fish per jar. Each diet treatment (PC, A and FL) consisted of three replicates, randomly assigned to the nine jars. All fish in each jar were measured and wet weights were determined at 7, 9 and 12 weeks. Food was withheld for 24 hours prior to sampling. Immediately before sampling, the fish were lightly anesthetized with tricaine methane sulfonate (50 ppm), and standard lengths were measured as described above. Each fish was then carefully wicked dry, transferred to a tared, covered 5-mL beaker of reconstituted water, weighed on a microbalance (to 0.01 mg), and then placed in jars filled with 1.5 L of aerated, reconstituted water to recover. Mortality associated with the above procedure was low (1.6 - 3.0 %) during the 12 weeks. Instantaneous growth rates (IGRs) (Weatherley and Gill 1987), based on natural log (Ln) of wet weight measurements, were determined for the replicates of each diet treatment from 0 to 7, 7 to 9 and 9 to 12 weeks by the formula:

$$\frac{(\text{Ln wet weight final} - \text{Ln wet weight initial})}{\text{\# days}} \times 100$$

Surviving fish at 12 weeks were used to establish adult growth, survival, and reproductive

success of broodstock under different diet treatments. The triplicate groups of fish from the respective diet treatments were pooled in 37-L acrylic aquaria in a recirculating system such that all aquaria received the same reconstituted water and were subjected to the same temperature and light regimes. The time to first egg production for females from each diet treatment was recorded.

At week 24, 18 fish from each treatment were randomly sampled for wet weight determinations. Of these, 15 individuals from each treatment were dried at 60°C for 24 h and dry weights determined. The remaining three fish were fixed in Davidson's solution, embedded in resin and stained with toluidine blue for histological evaluation. Additionally, 12 females and six males from the PC- and A-diet treatments were randomly selected and sorted into three broodstock groups (henceforth called "broodstock"), each consisting of 4 females and two males. The broodstock were housed in mesh-bottomed PVC cylinders in a recirculating system. The six broodstock containers were suspended in a random arrangement in two 100-L tanks, and identical rearing conditions were maintained.

Evaluation of broodstock reproductive success was conducted during weeks 29, 31 and 34. Eggs were collected over 3 days from each replicate broodstock group and incubated separately. Percent viable hatch was determined for replicates from both sample periods. Eggs collected during week 34 were evaluated for developmental abnormalities. Abnormal embryos were removed and incubated separately until they hatched or died. Hatchlings were evaluated for normal development; i.e., swim bladder inflation and normal swimming behavior (Marty, Nunez, et al. 1990a).

Trial 2

Newly hatched larvae with normal swim bladder inflation were pooled and equally distributed among ten 37-L acrylic aquaria, 250 larvae per aquarium. Diet treatments (PC and F/A) were randomly assigned to aquaria with 5 replicates per diet.

Ten fish per replicate were sampled from different regions in each aquarium every 10 days for wet and dry weight determinations as described for Trial 1. At 30, 60, 90 and 110 days, morphometric analyses of total length, maximum depth, and maximum width were conducted. Instantaneous growth rates were determined for wet and dry weights and for morphometric parameters (Weatherley and Gill 1987). Samples for general histology were taken at 20 day intervals. Fish were anesthetized, placed in Karnovsky's fixative, and processed routinely in paraffin for hematoxylin and eosin (H & E) staining.

At days 35, 70, and 110, 20 fish from each diet treatment were randomly selected for analysis of enzyme activity: Ethoxycoumarin 0-deethlyase (ECOD), Glutathione S-transferase (GST), and Gamma-glutamyl transferase (GGT). Visceral masses (day 35 only) or livers (day 70 and 110) were dissected free from anesthetized fish, pooled according to diet treatment, and frozen at -80°C. Samples were homogenized in cold sucrose buffer with a Potter/Elvehjem (Teflon/glass) homogenizer. The S9 fraction was prepared by centrifugation of the homogenate for 30 min at 9000 x g. Ethoxycoumarin 0-deethlyase, a mixed function oxidase enzyme, was estimated by the production of 7-hydroxycoumarin. The supernatant was assayed fluorimetrically for ECOD activity as described (Greenlee and Poland 1978). Glutathione S-transferase, a microsomal and cytosolic conjugating enzyme, was measured spectro-photometrically using chloro-dinitrobenzene (CDNB) as the substrate (Laurén, Halarnkar, et al. 1989b).

Gamma-glutamyl transferase (GGT), a cytosolic phase II enzyme found primarily in the kidney, was assayed using Sigma Kit 545-1 (Sigma Chemical Co., St. Louis, MO). Protein was quantitated by the method of Bradford (Bradford 1976).

Fish were monitored daily and the time to first egg production and the number of eggs produced by each replicate of each treatment were recorded. Reproductively active fish were removed from the treatment aquaria to serve as broodstock. These fish were placed in separate 37-L aquaria and maintained on their respective diet treatments. After acclimatization, eggs were collected from these broodstock over two periods of 5 days. Eggs obtained from the broodstock of each treatment group were incubated separately. and evaluated for developmental abnormalities and viable hatch as described in Trial 1.

Statistical Analysis: Data were analyzed according to statistical methods described by Sokal and Rohlf (Sokal and Rohlf 1981). All data presented as percent values were arc-sin transformed before analysis. In Trial 1, standard lengths, wet weights, and instantaneous growth rates of the larvae and juveniles were analyzed by one-way nested ANOVA. Differences were ranked by the Scheffe test. Percent hatch and survival were compared by the Student's t-test and one-way ANOVA, respectively. Data from Trial 2 were evaluated using the Student's t-test. A significance level of .05% was used throughout the study. Enzymatic data from Trial 2 were evaluated using the Spearman's rank correlation. A significance level of 0.5 was used for this nonparametric test.

Results:

Fish readily consumed all diets in both diet trials. Larvae began feeding approximately 24 to 48 hours after hatching, as noted by the presence of food particles in the gut. Medaka fed

at the water surface, as noted by Yamamoto (Yamamoto 1975), as well as at the bottom and in the water column.

Trial 1

Nutritional adequacy of the different diets was evaluated in terms of growth, survival, histology and reproductive success of medaka.

Survival and growth: Survival to 12 weeks was not significantly different in any of the diet treatments. Percent survival (mean ± SE) was as follows: PC-diet = 83.3 ± 4.0%; A-diet = 91.7 ± 7.7%; FL-diet = 80.0 ± 9.8%). Most deaths occurred within three weeks of hatching and these fish were notably smaller than their cohorts. Survival from 12 to 24 weeks was similar in both the PC- and A-diet treatments (80% and 83.3%, respectively). Survival of fish fed the FL-diet was much lower during this period (48.3%), and 76% of the surviving fish (22 of 29) were pale and emaciated. Necropsies of moribund and dead fish from the FL-diet group revealed depleted muscle tissue and fat deposits.

Significant differences in wet weights and standard lengths were noted between fish reared on the different diet treatments. Fish fed the FL-diet had significantly lower wet weights and standard lengths at 7, 9, and 12 weeks than fish fed either the PC- or A-diets (Figure 1). Average standard lengths of the fish fed PC- and A- diets did not differ significantly at 7, 9, or 12 weeks; however, corresponding wet weights of fish fed the A-diet were significantly greater. There were no significant differences in length of fish fed the PC-or A-diets (Figure 1). At 24 weeks, fish reared on the FL-diet had significantly lower wet and dry weights. Wet and dry weights were not significantly different between adults reared on the PC- or A-diets (Table 3).

Mean rates of change (i.e. growth) between sample periods were evaluated using IGRs.

Wet weight IGRs of all diet treatment groups were highest in the period from hatch to seven weeks and decreased over time (Table 4). This decrease in IGRs was significant in all three diet treatments. Fish fed the PC- and A-diets did not have significantly different wet weight IGRs from hatch to 7 weeks, however, IGRs of fish fed the FL-diet were significantly lower during this period. Wet weight IGRs were not significantly different between any of the diet treatments from 7 to 9 weeks or from 9 to 12 weeks.

Histology: Histological evaluation revealed no abnormalities in the internal organs of the fish reared on the PC- or A-diets; however, there was extensive muscle wasting in fish fed the FL-diet. Muscle bundles of these fish were atrophic and there were large spaces between adjacent muscle bundles. No other abnormalities were noted in the internal organs of the fish reared on the FL-diet.

Skeletal deformities: No spinal deformities were noted in medaka fed the PC- or A-diets, but one female in the FL treatment group had kyphosis.

Reproductive success: Medaka fed the A-diet were the first to become reproductively active with the first eggs appearing at 13 weeks posthatch. Females fed the PC-diet first produced eggs at 14 weeks. By 24 weeks, all of the females in the PC- and A-diet groups had been observed with eggs. Broodstock fed the A-diet produced eggs with a slight orange tint, whereas PC eggs were uncolored. Only 2 out of the remaining 29 FL-fed fish produced eggs (noted at 23 and 24 weeks). Due to the lack of reproductively active females from the FL-diet group, these fish were not used for further experiments evaluating reproductive success.

The number of eggs collected per female for evaluation of reproductive success ranged from 22 to 80 for PC-diet broodstock and 28 to 60 for A-diet broodstock. Percent viable hatch

did not differ significantly between diet groups (PC-diet: 94.4 ± 1.2%; A-diet: 94.4 ± 1.3%). Incidence of developmental abnormalities was low and did not differ significantly between diet treatments (PC-diet: 5.6 ± 2.4%; A-diet: 5.6 ± 1.9%).

Trial 2

Survival and growth: Survival to 110 days was not significantly different in either of the diet treatments (PC-diet 95.0 ± 0.5%; F/A-diet 96.7 ± 0.5%). Fish fed the F/A-diet had significantly greater mean wet weight, total length, and maximum depth at all sample points after day 0 (Figure 2; Table 5). The highest IGRs, calculated from wet weight, occurred during the interval between hatch and 30 days for both diet treatments. Instantaneous growth rates of the F/A treatment, however, were significantly higher than those of the PC treatment during this period (Figure 3). Between 30 and 40 days, IGRs were significantly higher in fish fed the PC-diet. After 40 days, IGRs were not significantly different between diet treatments, except between 90 to 100 days.

Analyses based on morphometric parameters of total length, maximum depth, and maximum width (Table 5) showed fish fed the F/A-diet had significantly greater IGRs for all morphometric parameters for the interval of 0 to 30 days (Figure 4). However, between 30 and 60 days the PC-diet fed fish showed significantly greater IGRs for total length, maximum width and total depth. Maximum depth and maximum width IGRs were also significantly greater in the PC treatment group from 60 to 90 days. There was no significant difference in IGRs between diet treatments after 60 days (total length) or 90 days (maximum width and depth and total length).

Liver Histology: No differences in liver lipid or glycogen were seen between the F/A-diet

and the PC-diet. In both diet groups, we observed occasional, pale, rounded hepatocytes with brightly eosinophilic cytoplasmic bodies and nuclear eosinophilia with marginated chromatin. These degenerating and necrotic hepatocytes, classified as apoptotic cells, appeared in similar numbers in F/A fed fish (3 of 51 livers) and PC-diet fed fish (3 of 49 livers). Apoptosis occurred as early as 40 days posthatch and continued sporadically through the end of the study (110 days posthatch).

Liver Biochemical Parameters: The ECOD and GST activities for the two feeding regimes were similar except for on day 110 where GST activity appeared higher (significant to 95% level; Spearman Rank correlation) in fish fed the PC-diet (Table 6). Both the F/A- and PC-diet groups at 35 days had detectable levels of GGT (12.45 and 12.74 nmol/min/mg protein respectively). Activity of GGT was not detectable at 70 or 110 days in fish fed either diet.

Skeletal Deformities: Skeletal deformities were found in 67 adult fish from the F/A-fed groups (average from all aquaria = 5.4%). Gross lesions ranged from slight axial deformation to multiple lateral (scoliosis) and dorsoventral (lordosis/kyphosis) curvatures (Figure 5), as well as mild cranial abnormalities. The dorsoventral curvature, while difficult to ascertain grossly, seemed to occur between the second and third vertebrae. Radiographically and histologically, spinal deformities were more obvious. The F/A-diet-related vertebral abnormalities were less severe than the congenital "wavy tail" abnormality (not observed in this study) that has been previously described (Yamamoto 1963). Only one fish with a very slight axial deformation was found in the PC-diet fed group during the final count (0.08% incidence).

Reproductive success: Time to first egg production was not significantly different between diet treatments (mean F/A-diet= 92 ± 3.8 days; mean PC-diet= 90 ± 2.2 days). There were no

significant differences between the total number of eggs (PC-diet= 156 ± 19.2 ; F/A-diet= 144 ± 48.35) or the numbers of fertilized (PC-diet= 141 ± 19.1; F/A-diet= 122 ± 43.8) and unfertilized eggs (PC-diet= 15 ± 1.2; F/A-diet= 22 ± 4.9), produced from the onset of egg production to 110 days in either diet treatment. At 110 days, however, the proportion of fish which did not show external sexual differentiation was significantly higher in the PC-diet group (34.1 ± 2.0%) compared to the F/A-diet group (15.5 ± 1.1%). Percent viable hatch was similar in both diet treatment groups (pooled mean PC-diet = 85.1%; pooled mean F/A-diet = 83.7%), and incidences of developmental abnormalities were low (pooled mean PC-diet = 0.95%; pooled mean F/A-diet = 0.90 %) for offspring.

Discussion

Despite the use of different rearing systems in Trials 1 and 2, results of this study have demonstrated the overall nutritional adequacy of the PC-diet. Adequate growth and development, with no deleterious effects, resulted when medaka were fed with a regime comprised solely of the purified diet. The PC-diet meets the recommendations of the Consensus Committee (Consensus Committee 1984). Its use provides a way to decrease experimental variables by helping to define the dietary needs of medaka while obviating the use of live food. The PC-diet is suitable for maintaining medaka from first feeding to reproductive maturity, and provides a standardized, nutritionally adequate and consistent alternative to conventional diets.

Potential long-term problems may result when medaka are fed a conventional diet (FL- or F/A-diets). The incidence of skeletal deformities in the F/A group (Trial 2) appears to be diet related, because such alterations were not observed in the PC-diet fed group. It is unlikely that skeletal deformities were the result of water-borne contaminants (Couch, Winstead, et al. 1977)

or aquarium/system factors because medaka in both diet treatments were reared under identical conditions in the same recirculated water. Genetic factors were not likely to be responsible for skeletal alterations in this study because medaka were randomly selected from identical broodstock. A heritable recessive trait known as "wavy tail" exists in medaka (Yamamoto 1963); however, this external characteristic, apparent at hatch, was not seen in larvae initially selected in our study.

The FL-diet alone does not appear to meet the nutritional requirements of medaka. Poor growth, reduced survival, and emaciation were noted soley in fish fed the FL-diet (Trial 1) and reproductive success was low in this diet treatment. The connection between poor growth and survival, and nutritionally inadequate or imbalanced feeding regimes is well documented (Roberts and Bullock 1989), whereas the influence of nutritional status and reproductive success may vary between species (Blaxter 1988). In medaka, however, inadequate broodstock nutrition may compromise fecundity or maternal somatic growth (Hirshfield 1980).

Reproductive success (ie. fecundity and egg hatchability) and larval viability did not differ significantly between medaka fed the PC-diet and fish fed A-diet (Trial 1) or F/A-diet (Trial 2). Additionally, in Trial 2, growth rates (as IGRs) of PC-diet broodstock were comparable to, or higher than, broodstock fed the F/A-diet after day 30 . These results indicate that the nutritional status of PC-diet broodstock was not compromised such that somatic growth was sacrificed for egg production.

Coloration was the only difference between eggs of broodstock fed the diets containing live food (A- and F/A-diets: orange eggs) and those from medaka fed the PC-diet (colorless eggs). Egg pigmentation is derived from (Craik 1985; Harris 1984; Mommsen and Walsh 1988;

Schaeffer, Tyczkowski, et al. 1988) and appears to be proportional to levels of carotenoids in the maternal diet (Harris 1984). Takeuchi (Takeuchi 1960) showed that female medaka fed a carotenoid-free diet produced colorless eggs. The PC-diet, without carotenoids, produced similar results.

Our results indicate that reproductive success or subsequent embryo and larval viability in medaka are not affected by an absence of carotenoids in the PC-diet. The role of carotenoids in reproductive success and embryo viability of fish is speculative, remaining unsubstantiated by controlled laboratory experiments (Choubert 1986; Tacon 1981; Watanabe, Itoh, et al. 1984). While studies show egg yolk carotenoids are a source of chromatophore pigment in newly hatched larvae (Mommsen and Walsh 1988; Steven 1949), absence of chromatophore pigments apparently does not affect larval survival or viability. Torrissen (Torrissen 1984) found no effect of egg carotenoid level on embryo and alevin survival in Atlantic salmon. Similarly, Harris (Harris 1984) and Dabrowski *et al.* (Dabrowski, Luczynski, et al. 1987) were unable to correlate broodstock reproductive success with dietary carotenoid.

Liver histological and biochemical changes were observed during growth in juvenile fish. Apoptosis, seen in both the F/A and PC treatments, is a transient alterations that may reflect tissue remodeling (Wyllie, Kerr, et al. 1980) as part of generational growth-related phenomena. Livers of medaka from both diet treatments showed detectable levels of ECOD and GST activity. This indicates that both diets provide adequate nutrition for development of the xenobiotic metabolizing enzymes necessary for detoxification and activation of endogenous and foreign compounds. Both ECOD, a good indicator of the constitutive level of cytochrome P-450 monooxygenase activity, and GST, a conjugating enzyme, increased from day 75 to day 110.

Increases in P-450 and Phase 2 conjugating enzymes have been documented to increase during development in rodents (Zongzhu, Sato, et al. 1982) but this is the first citing of this increase in medaka. This is important because changes in metabolism with age of the animal may affect its response to toxicants (Zongzhu, Sato, et al. 1982). The presence of GGT activity at day 35 but no later, may represent remnants of GGT isozymes, and in this way, be analogous to rodents where activity is fetal and neonatal only (Fiala, Fiala, et al. 1972). Alternatively, livers at day 35 were so small that dissection was very difficult and assays were conducted on visceral masses. Visceral masses frequently contain kidney which is generally rich in GGT at all life stages (Fiala, Fiala, et al. 1972). On day 110 post-hatch, higher GST levels in PC-fed fish were observed. Glutathione S-transferase is used in protection from toxic injury (Kaplowitz 1980) and the higher levels of GST in PC-fed fish may reflect the enhanced nutritional status of these fish. It is unlikely a water-borne effect, since the same recirculated water was used with both diet treatment groups.

Although the present formulation of the PC-diet appears to be nutritionally adequate, the significant lag in early growth rates (ie. 0-30 days), and resultant differences in weight and morphometric parameters need to be addressed. Superior growth of fish fed live food versus formulated diets has been well documented in other species and seems especially apparent during the larval stage (Appelbaum 1985; Dabrowski, Charlon, et al. 1984; Dabrowski and Kaushik 1985; Dabrowski and Poczyczynski 1988a; Dabrowski and Poczyczynski 1988b; Jobling 1986; Lauff and Hofer 1984). Several hypotheses have been proposed including: (i) preference of first-feeding larvae for live and motile food compared to inert food (Appelbaum 1985); (ii) auto-digestion of previously live food in the gut, resulting in breakdown of complex nutrients to

simpler molecules more readily assimilated by larval fish, which may lack fully developed digestive systems (Dabrowski, Charlon, et al. 1984; Dabrowski and Kaushik 1985; Dabrowski and Poczyczynski 1988a; Dabrowski and Poczyczynski 1988b; Lauff and Hofer 1984); and (iii) overload of the digestive and absorptive capacity of the larval gut when fed (comparatively) high energy, formulated feeds (Jobling 1986).

Leaching of water-soluble nutrients from the PC-diet prior to consumption may be a factor contributing to lower initial growth rates. Cloudiness of the water immediately surrounding diet particles was noted after the PC-diet was first added. This could indicate leaching which would lower the quality of food or promote bacterial overgrowth. Poor visual acuity and prey capture efficiency by larval medaka may add to this problem. Although medaka larvae are actively free-swimming and able to capture prey within 24 hours of hatching, their visual acuity is not fully developed until approximately 3 weeks of age (Ohki and Aoki 1985).

Although the delay in initial growth of medaka fed the PC-diet was reflected in lower wet and dry weights (Trials 1 and 2), the fish were able to compensate for these differences. In Trial 1, there was no significant difference in wet or dry weights at 24 weeks, and we have subsequently found that Trial 2 fish maintained on either diet treatment to 35 weeks posthatch did not differ significantly in wet or dry weights. This "recovery growth" pattern exhibited by medaka fed the PC-diet is similar to that noted for rainbow trout fingerlings starved and then fed *ad libitum* (Weatherley and Gill 1981). In the rainbow trout, as in medaka, there were no deleterious effects of this early lag in growth. For medaka reared on the PC-diet, development to maturity, as well as hatchability or viability of offspring were not compromised.

The undefined lipid portion (soy lecithin, corn and cod liver oil), of the PC-diet remains

an area of concern. Adventitious dietary xenobiotics, which may modulate toxicity (Leatherland and Sonstegard 1982; Leatherland, Sonstegard, et al. 1979; Malins, McCain, et al. 1988; McCain, Brown, et al. 1988), may be introduced with this component. Initial attempts to replace the lipid portion of the PC-diet with purified fatty acids were not successful (DeKoven 1990); however, we continue to pursue this aspect of medaka nutrition.

Although further refinements are needed in the PC-diet, it has distinct advantages over conventional diets. It is more nutritionally consistent and does not require separate culture of food items; hence, it is much less labor intensive than feeding live foods. It is also less likely to contain the range of xenobiotics possible in whole, live food. As a standardized, purified diet, the PC-diet would decrease variation in results both between testing laboratories and in serial studies within a given laboratory. The PC-diet is also a good vehicle for delivering known amounts of experimental toxicants, a complex and questionable ambition using live food. Finally, because of the purified, open formulation of the PC-diet, it can be used to evaluate dietary modulation of toxicity through manipulation of amounts and types of selected nutrients. This opens a new area of research with medaka, one which is not possible with live foods or commercial closed-formula diets presently in use.

Chapter 2

Comparison of Hepatic Neoplasm Frequency in Medaka Purified Casein Based or Conventional Diet

Background

Progress to date has shown that the purified casein diet (PC) is capable of supporting medaka from hatch through egg production and that the progeny of adults fed only this diet are healthy fish. This accomplishment has largely met the initial objective of this contract. Now we moved to the next major objective which was to compare tumor incidence (frequency) between medaka fed a conventional ration (Tetramin flakes, Tetrawerke, Germany) plus two days supplementation each week with brine shrimp (*Artemia sp.*) nauplii and those fed the PC diet.

At present, medaka under carcinogen bioassay at various laboratories receive a variety of different diets. We have consulted with Dr. Keith Cooper at Rutgers University, their laboratory typically feeds Tetramin fiakes alone or with brine shrimp supplementation. The United States Environmental Protection Agency, Environmental Research Lab at Duluth, Minnesota feeds medaka under test brine shrimp nauplii only (Personal Communication from Dr. Rodney Johnson). The Gulf Coast Marine Research Laboratory feeds day-old hatchlings an infusoria of live organisms suitably sized for uptake by young medaka. This initial feeding is continued for a period of approximately two weeks after which the fish receive brine shrimp nauplii and are eventually moved to a commercially available ration supplemented with ocean plankton (Personal Communication from Dr. W. Walker).

The formulation of commercial diets are typically proprietary and constituents may vary

from batch to batch. Even the components may be designed more for production than for rigid tests needed to compare nutritional modulation of carcinogenesis or to provide the consistency between tests which are needed for statistical evaluations. When natural (live) foods are used, the possibility of addition of adventitious xenobiotics arises.

We hypothesize that xenobiotics might alter the post-initiation phases of carcinogenesis (i.e., promotion and progression). It is entirely possible that the time to tumor, as well as the tumor frequency, will vary as a function of the diet. We therefore ultimately plan to use two different carcinogens under different times and routes of exposure and to determine the neoplastic potential of each. To meet this goal, very young medaka (21 days post-hatch) are briefly (48 hours) exposed to an aqueous solution of 350 ppm of diethylnitrosamine (DEN). Serial bioassay at monthly intervals was then used to determine the frequency of tumor formation. In the second experiment which has not yet been initiated, fish fed either the PC or flake plus *Artemia* (F/A) diet will be reared until sexual maturity. At this time they shall be exposed to their original diet to which aflatoxin B_1 has been added. Using the various diets as a carrier vehicle, the frequency of liver tumor formation will be determined following prolonged dietary exposure. In this chapter, we report on our progress through seven months comparing tumor frequency in medaka fed the PC diet or the flake plus *Artemia* (F/A) diet from day one of hatch and exposed to a brief pulse of DEN at 21 days.

Materials and Methods

Fish

Broodstock of golden variety medaka were maintained at 25°C under a 16 L:8D photoperiod. Eggs from several females were pooled and incubated in modified (no methylene

blue), aerated, embryo rearing medium (Kirchen and West 1976; Rugh 1962) at 25±1°C. Medium was replaced daily at which time any dead embryos were removed. Hatch took place at 9 to 10 days after fertilization. All fish were reared in a recirculation aquarium system using reconstituted water prepared following EPA guidelines (Horning and Weber 1985) for moderately hard water. Reconstituted water was prepared in batches of 500 gallons using reverse osmosis produced water as a feed. Water temperature was maintained at 25±1°C. Ammonia and nitrite levels were monitored weekly and maintained at less than 0.1 ppm. Nitrates, pH, conductivity and hardness were monitored weekly. Dissolved O_2 was maintained at or near saturation.

Newly hatched larvae with normal swimbladder inflation were randomly sorted and equally distributed among aquaria. To reduce bias in selection of fish, a 10-inch wide net was used to trap and concentrate approximately 100 fish. From this pool, and while the fish remained immersed, individuals were collected with a glass beaker and randomly assigned, in series, to the aquaria. The process was repeated serially until the desired number of fish was obtained for each aquarium. This process permitted selection of healthy individuals and overcame bias due to capture/evasion effect. An assistant was given 4 identically sized, sealed envelopes of uniform appearance and asked to place one on each aquarium. A card, inside each envelope, designated the diet for fish within that aquarium. Fish were fed to slight excess twice daily and tanks were siphoned to remove excess food and feces.

At 21 days of age, normal and healthy juveniles, of uniform size, (500 fish per treatment) were selected as described above. Also, 100 fish were selected from each diet for the control group at this time. Abnormal or weak fish were discarded. Selected fish, in labeled containers corresponding to diet group, were then transported to our exposure facility.

Medaka from each diet group scheduled for exposure to diethylnitrosamine (DEN) were placed in 10-L glass aquaria (density = 50 fish/L) and exposed for 48 hr to an aqueous bath of 350 ppm DEN. Actual concentrations of DEN in exposure water were determined at 0, 12 and 24 hours. After this, total replacement with fresh reconstituted water plus DEN (350 ppm) was done. Similarly, assay for DEN concentration was done immediately after mixing in the aquaria and at 36 and 48 hours. Except for DEN, control fish were subjected to the same conditions as the exposed fish and placed in a glass aquarium with 2-L of reconstituted water (density = 50 fish/L). After exposure, fish were transferred to clean reconstituted water and then feeding with their respective previous diets was initiated. A brief duration was selected in order to insure homogeneity of DEN concentration within aquaria. This regime has been used in our laboratory to produce hepatocellular carcinoma in medaka. The brief initiation followed by somatic and liver growth, considered as promotional factors, enables a more thorough comparison of the effect of diet on promotion and progression of hepatic neoplasms. Aquarium water was tested for residual DEN, when none was detected fish were transferred to the recirculating system in our rearing facility. Exposed fish were then placed in two 20-gallon aquaria, 250 fish per aquarium. Control fish were placed in one 10-gallon aquarium. Fish were allowed to "grow out" under the same conditions and previous diet regimes. Selection of rack and placement within racks for the 4 aquaria containing exposed fish was done in a randomized fashion using sealed envelopes as described above.

Quantification of DEN was by spectrophotometric analysis at a wavelength of 230 nm (IARC, 1981), of water taken directly from the aquaria. Standards were prepared with reconstituted water and ranged from 0.1 to 10 ppm. By direct comparison with gas

chromatography, our previous work had shown that the spectrophotometric method has proven to be reliable and faster.

Particle sizes of the formulated rations were increased as the fish grew. From hatch to 4 weeks, fish were fed formulated rations ranging from 100 to 250 µm particle diameter. After 4 weeks, the fish were fed particle sizes ranging from 250 to 850 µm diameter. Newly hatched brine shrimp were separated from unhatched and empty cysts and rinsed with distilled water prior to being fed to fish (*Artemia* F/A group only). *Artemia* F/A group was fed flaked food five days per week and brine shrimp nauplii two days (Tuesday and Friday) each week. Fish in the other two aquaria were fed only the purified casein (PC) diet (De Koven et al, 1992).

Each month, fish were randomly sampled (10 per replicate, total of 20 per treatment-exposed fish; 4 per treatment-control group) for wet weight determinations and general histology. Collected fish were anesthetized in MS-222, placed in Bouin's fixative for 48 h, dehydrated in graded alcohol solutions and processed for paraffin embedment. Sections of paraffin embedded material were cut at 6 µm and stained with hematoxylin and eosin (H&E). Histologic analysis was performed to enumerate tinctorially altered foci, adenoma, cholangioma, hepatocellular carcinoma, cholangio-cellular carcinoma and mixed cell (both hepatocytes and biliary epithelial cells) tumors. The above histologic methods closely followed procedures which have been used in this lab in previous studies (Hinton, Lantz, et al. 1987; Laurén, Teh, et al. 1990).

Results and Discussion

Growth: Both control groups showed greater growth than their respective exposed groups (Tables 7a and b and Figure 6). Although the changes in weight have been monitored through

180 days, fish exposed for 48 hrs to DEN continue to show less growth than controls and the clustering of the two exposed diet treatments (Figure 6) suggests that it is DEN and not the particular diet which is responsible for the altered growth pattern. By comparison, PC diet fed controls showed a trend toward greater growth during the 90-150 day interval than did the control medaka fed the flake *Artemia* regimen (Figure 6).

Mortality: Two DEN-exposed fish, one from each diet group, died during the 48 hr exposure. No control deaths were recorded during the 48 hr period (Table 8a). Subsequently, 95 fish (19% mortality) died in the PC-fed and DEN-exposed group. Apparent lower mortality (60 fish, 12%) was seen in the flake *Artemia*-fed DEN-exposed group (Table 8b). The histogram (Figure 7) graphically represents actual numbers of dead fish in individual "grow out" aquaria. We observed no "tank-specific" effect. Mortality data suggest that both groups are showing DEN-induced toxicity with the PC diet group showing possible greater effect.

Histopathology: Control hepatic alterations were minimal throughout the study. After the initial 48 hrs of test, controls corresponding to this time interval showed no lesions. After 1 month, a single PC diet-fed control fish showed focal perivascular necrosis and spongiotic change. No lesions were seen in the F/A-fed control fish at one month. At two months, a single F/A-fed fish showed a single vacuolated focus similar to that shown in Figures 8 and 9. Similarly, two PC-fed control fish showed spongiotic change. At month three, PC-fed controls showed focal spongiotic change (3 of 4 examined). F/A-fed controls showed no lesions at month three. In month four, one F/A-fed control showed focal spongiosis hepatis. Corresponding PC-fed controls were lesion free. All control fish from months five, six and seven were lesion free. Spongiosis hepatis has been reported in carcinogen-exposed rats (Bannasch, Bloch, et al.

1981) and in medaka (Hinton, Lantz, et al. 1984a) and *Cyprindon variegatus* (Couch 1991). The lesion has also been reported at low incidence in multi-year control fish. In the present study, the lesion may be involved in growth-related hepatic remodeling (Wyllie, Kerr, et al. 1980). Except for the single vacuolated focus (in flake *Artemia*-fed control at month two), control livers have been free of foci and neoplasms.

Classification Nomenclature - Hepatocellular Neoplasms and Early Lesions

There are no published guidelines for nomenclature of fish, specifically medaka, hepatic neoplasms and associated lesions. Rather, a collection of various terms and lesion descriptions exists usually as a small section in each of the original papers. Rodent bioassays have adopted uniform criteria by which alterations are classified (Boorman, Eustis, et al. 1990; Maronpot, Montgomery Jr, et al. 1986). The adoption of such criteria, while facilitating uniformity of bioassay results, must reflect the breadth and nature of histopathologic alterations. I have contributed a chapter on normal morphology and early fish hepatic alterations encountered after laboratory exposure to carcinogens (Hinton, In Press:a). This laboratory has contributed much to our understanding of histopathologic biomarker lesions (Hinton, In Press:b; Hinton and Couch 1984; Hinton, Lantz, et al. 1987; Hinton and Laurén 1990a; Hinton and Laurén 1990b; Hinton, Walker, et al. 1984b; Hinton et al. 1992). In response to needs expressed at the 1991 Histopathologic Workshop on liver lesions of fishes exposed to carcinogens, United States Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida, and at the recent National Toxicology Program meeting on Fish Carcinogenic Models, we have devoted efforts under this contract to describe and illustrate medaka liver histopathology associated with DEN-induced carcinogens. Table 9 presents key features of the medaka

classification system for foci and neoplasms, the principal lesions of this study. Only those lesions visible with H&E staining were enumerated.

Regardless of the diet, DEN-exposed fish developed foci and neoplasms further supporting our earlier studies with this compound in medaka (Hinton 1989a; Hinton, Couch, et al. 1988b; Hinton, Hampton, et al. 1985; Hinton, Laurén, et al. 1988a; Hinton, Teh, et al. In Press). Alterations encountered after medaka are exposed to DEN include an early hepatotoxicity primarily directed at hepatocytes. Lesions of an early toxic nature characterized all DEN-exposed fish of this study. These have been described in detail at both the light and electron microscopic levels (Braunbeck, Teh, et al. In Press; Laurén, Teh, et al. 1990).

The foci and neoplasms of this study followed the early toxic response. After one month, 14 foci were seen in PC-fed DEN-exposed medaka (1 basophilic, 4 clear cell and 9 vacuolated). Corresponding F/A-fed fish showed a total of 9 foci (5 basophilic and 4 vacuolated). After two months, PC diet fish showed 18 foci (2 eosinophilic, 2 clear cell and 14 vacuolated). In F/A-fed fish, 20 foci were seen at this time (3 eosinophilic, 2 clear cell, 15 vacuolated). By the end of 3 months, PC-fed fish showed 11 foci (2 basophilic, 4 clear cell, 5 vacuolated). At this time, F/A-fed fish showed 35 foci (1 basophilic, 2 clear cell and 32 vacuolated). Month four fish showed 10 foci (PC diet; 5 basophilic, 3 eosinophilic, 9 clear cell, 2 vacuolated). F/A-fed fish at this month showed 21 foci (1 basophilic, 4 eosinophilic, 16 vacuolated). At month five, PC-fed fish showed a total of six foci (1 eosinophilic, 3 clear cell and 2 vacuolated). Month five F/A-fed fish showed 16 foci (6 eosinophilic, 2 clear cell and 8 vacuolated). Month six medaka fed PC diet showed 40 foci (3 basophilic, 5 eosinophilic, 1 clear cell and 21 vacuolated). Corresponding F/A-fed fish showed a total of 9 foci (2 eosinophilic and 7 vacuolated). In month

7 there were 37 foci in PC-fed medaka (8 basophilic, 7 eosinophilic, 2 clear cell and 20 vacuolated). F/A-fed fish at month 7 showed 25 foci (4 basophilic, 4 eosinophilic, 14 clear cell, 3 vacuolated). Statistical evaluation of the data will follow the final sampling for histopathologic analysis and does not fall within the first half of this project.

The foci enumeration should be regarded as semiquantitative since no morphometric procedures (Weibel 1980) were followed. These are to be done in the remaining two years. However, foci preceded neoplasms suggesting similarity in progression between rodent (Farber 1976; Pitot 1983) and medaka (Hinton, Couch, et al. 1988b) hepatocarcinogenesis. Of the medaka foci, basophilic and eosinophilic are generally regarded as the most relevant to tumorigenesis. A basophilic focus found in a liver section from a F/A-fed, DEN-treated, female medaka at six months after onset of exposure is shown in figure 8. The major difference of cells within the focus versus their counterparts in the "noninvolved liver" are related to the staining within the cytoplasm. The architectural arrangement of surrounding liver and focus is nearly identical with the latter perhaps showing slight enhancement of the tubular arrangement (fig. 8). Figure 9 illustrates feaures of an eosinophilic focus. This particular focus was seen in the liver section of a female medaka fed the PC diet for 6 months. Component hepatocytes of eosinophilic foci differ appreciably in size (fig. 9). Features of a clear cell focus from the liver of a female medaka at 5 months after the onset of exposure are seen in figures 10 and 11. This fish was fed the PC diet. Cells of clear cell foci show the least staining over cytoplasm in H&E stains. They are followed by normal, glycogen enriched cells and then by the cells of eosinophilic and basophilic foci. Abrupt margins where cells with focal staining characteristics abut on cells with normal features characterize most foci (figs. 8,9,10,11). A vacuolated focus

is shown in figure 12. This lesion was seen in the liver of a PC fed, female fish at 7 months after the onset of exposure. By contrast with figures 10 and 11, the large vacuoles of smooth outer contour and eccentric nuclei differ from the appearance of clear cells. Vacuolated cells are regarded as fat filled hepatocytes. No diet specific differences among a single category of focus were encountered. The fate of the numerous vacuolated foci needs attention. Their occurrence in both control and treated livers is akin to certain "spontaneous" foci in rodent liver (Pitot, Campbell, et al. 1989). Our future work will be to quantify initial foci number and then to follow growth of these lesions. Once data is derived on number and growth, we can proceed with testing to determine initiation and promotion indices (Pitot, Campbell, et al. 1989) for individual compounds and complex mixtures.

Resultant neoplasms, by dietary group, are presented in sequential fashion (Table 10). Both rations proved sufficient to support tumor formation in DEN-exposed medaka. However, it appears that greater tumor yields may result from use of the F/A ration. Whether this is related to progressional and/or promotional factors not present in the PC diet remains to be tested. The time to first tumor appears to be identical whether fish were fed PC or F/A diets (Table 10). However, from 3 months on, a total of 10 and a total of 19 tumors resulted in PC and F/A groups, respectively (Table 10). It is too early to tell whether tumor promotional and/or progressional agents are present in the F/A ration. Complete statistical analysis will follow at the termination of the study.

Histopathologic analysis of a male medaka exposed to DEN for 48 hrs (350 ppm) and fed the F/A diet revealed the presence of a cholangioma at 6 months after onset of exposure (Fig. 13). Architecture of the biliary passageways retains a differentiated state. However, the profiles

of ducts are very numerous in this lesion. In addition, the fairly typical nuclei are beginning to "pile up" in multiple rows. There is no evidence that the neoplasm has invaded the adjacent parenchyma.

A cholangiocarcinoma is shown (Figure 14). This neoplasm was found in the liver of a male medaka fed the F/A diet. The neoplasm was encountered at 4 months after the onset of exposure to DEN (48 hr bath at 350 ppm concentration). By contrast with figure 13, the cholangiocarcinoma has invaded the adjacent parenchyma. Both ductular and trabecular patterns are indicated. Numerous mitotic figures are present (Figure 14).

A mixed hepato- and cholangiocellular tumor is shown in figure 15. This neoplasm was detected in the liver of a female medaka at three months after the onset of exposure to DEN. This particular neoplasm was predominantly cholangiocellular with hepatic parenchymal tubules between ductular elements. Elements of a solid hepatocellular carcinoma are shown in figures 16 and 17. In the low magnification view (Figures 16), the solid features and basophilic staining contrast with the remainder of the liver. Under higher magnification, nuclear pleomorphism and transformation of tubules into broad sheets of tumor cells are apparent (Figure 17). This tumor was detected at seven months after onset of exposure and was in the liver of a male medaka fed the F/A diet.

At seven months after onset of exposure, a female medaka fed the F/A diet was shown to have developed a large hepatocellular carcinoma (Figures 18 and 19). This lesion occupied the majority of the liver section and had a large necrotic component in its center (Figures 18 and 19). Tumor also contained foci of spongiosis hepatis (Figure 19).

Data through this midterm point indicate that the PC diet will prove adequate as a single

ration for medaka. The consistency of the open-formula, purified diet now makes it possible for us to pursue modulatory effects of diet and environment on fish liver carcinogenesis.

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REFERENCES

1. Affandi R, Biagianti S. (1987). A study of the liver of eels kept in captivity: disturbances in hepatocytes by artificial diets. *Aquaculture* 67:226-228.

2. Amat F, Hontoria R, Navarro JC. (1987). Preliminary nutritional evaluation of different *Artemia* nauplii as food for marine fish and prawn larvae. In: *International study on Artemia XLIV. Artemia research and its applications. Vol. 3. Ecology, culturing, use in aquaculture*, Sorgeloos P, Bengston DA, Decleir W et al (eds). Universa Press, Wetteren, Belgium, pp. 425-436.

3. Andersson T, Koivusaari U, Forlin L. (1985b). Xenobiotic biotransformation in the rainbow trout liver and kidney during starvation. *Comp. Biochem. Physiol.* 82C:221-225.

4. Ankley GT, Blazer VS. (1988). Effects of diet on PCB-induced changes in xenobiotic metabolism in the liver of channel catfish (*Ictalurus punctatus*). *Can. J. Fish. Aquat. Sci.* 45:132-137.

5. Aoki K, Matsudaira H. (1977). Induction of hepatic tumors in a teleost (*Oryzias latipes*) after treatment with methylazoxymethanol acetate. *J. Natl. Cancer Inst.* 59:1747-1749.

6. Aoki K, Matsudaira H. (1981). Factors influencing tumorigenesis in the liver after treatment with methylazoxymethanol acetate in a teleost, *Oryzias latipes*. In: *Phyletic Approaches to Cancer*, Dawe CJ, Harshbarger JC, Kondo S et al. (eds). Japan Scientific Society Press, Tokyo, pp. 205-216.

7. Aoki K, Matsudaira H. (1984). Factors influencing methylazoxymethanol acetate initiation of liver tumors in *Oryzias latipes*: carcinogen dosage and time of exposure. *Natl. Cancer Inst. Monogr.* 65:345-354.

8. Aoki K, Matsudaira H. (1986). Modification of methylazoxymethanol acetate initiated hepatocarcinogenesis in a teleost, *Oryzias latipes*. 2nd International Conference on the Combined Effects of Environmental Factors 1986. Kyoei Co., Ltd, Kanazawa, Japan. pp. 978-990.

9. Appelbaum S. (1985). Rearing of the Dover sole, *Solea solea* (L.), through its larval stages using artificial diets. *Aquaculture* 49:209-221.

10. Bac N, Biagianti S, Brusle J. (1983). Etude cytologique ultrastructurale des anomalies hepatiques du Loup, de la Daurade et de l'Anguille, induites par une alimentation artificielle. *IFREMER, Actes de Colloques* 1:473-484.

11. Bailey GS, Hendricks JD, Nixon JE, Pawlowski NE. (1984a). The sensitivity of rainbow trout and other fish to carcinogens. *Drug Metab. Rev.* 15:725-750.

12. Bannasch P, Bloch M, Zerban H. (1981). Spongiosis hepatis, specific changes of the perisinusoidal liver cells induced in rats by N-nitrosomorpholine. *Lab. Invest.* 44:252-264.

13. Beck AD, Bengston DA, Howell WH. (1980). Nutritional value of five geographical strains of *Artemia*: effects on survival and growth of larval Atlantic silverside *Menidia menidia*. In: *International study on Artemia Vi. The brine shrimp Artemia. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O et al (eds). Universa Press, Wetteren, Belgium, pp. 249-259.

14. Blaxter JHS. (1988). Pattern and variety in development. In: *Fish Pathology Vol. XIA*, Hoar WS, Randall DJ (eds). Academic Press, San Diego, pp. 1-58.

15. Boorman GA, Eustis SL, Elwell MR, Montgomery Jr CA, MacKenzie WF. (1990). Pathology of the Fischer Rat: Reference and Atlas, Academic Press, Inc., San Diego,

16. Bradford M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of proten utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

17. Braunbeck TA, Teh S, Lester SM, Hinton DE. (In Press). Ultrastructural alterations in hepatocytes of medaka (*Oryzias latipes*) exposed to diethylnistrosamine. *Toxicol. Pathol.*

18. Briggs JC, Egami N. (1959). The medaka (*Oryzias latipes*). A commentary and a bibliography. *J. Fish. Res. Board Can.* 16:363-380.

19. Bunton TE. (1990). Hepatopathology of diethylnitrosamine in the medaka (*Oryzias latipes*) following shorth-term exposure. *Toxicol. Pathol.* 18:313-323.

20. Choubert G. (1986). Pigments carotenoides et reproduction des poissons. *Bull. Fr. Peche Piscic.* 300:25-32.

21. Conklin DE, D'Abramo LR, Bordner CE et al. (1980). A successful purified diet for the culture of juvenile lobsters: the effect of lecithin. *Aquaculture* 21:243-249.

22. Consensus Committee. (1984). Summary and recommendations: A consensus report. *Natl. Cancer Inst. Monogr.* 65:397-404.

23. Couch JA. (1991). Spongiosis hepatis: Chemical induction, pathogenesis, and possible neoplastic fate in a teleost fish model. *Toxicol. Pathol.* In press:

24. Couch JA, Winstead JT, Goodman LR. (1977). Kepone-induced scoliosis and its histological consequences in fish. *Science* 197:585-587.

25. Cowgill UM, Emmel HW, Boggs GU et al. (1987). Variations in chemical composition of *Artemia* cysts from three geographical locations. In: *Artemia research and its applications. Vol. 1. Morphology, genetics, strain characterization, toxicology*, Sorgeloos P, Bengston DA, Decleir W et al (eds). Universa Press, Wetteren, Belgium, pp. 173-188.

26. Craik JCA. (1985). Egg quality and egg pigment content in salmonid fishes. *Aquaculture* 47:61-88.

27. Dabrowski K, Charlon N, Bergot P et al. (1984). Rearing of coregonid (*Coregonus schinzi palea* Cuv. et Val.) larvae using dry and live food. I. Preliminary data. *Aquaculture* 41:11-20.

28. Dabrowski K, Luczynski M, Oziczuga B et al. (1987). Relationship among coregonid fish reproductive effort, carotenoid content in eggs and survival of embryos. *Arch. Hydrobiol. (Suppl.)* 79:29-48.

29. Dabrowski KR, Kaushik SJ. (1985). Rearing of coregonid (*Coregonus shinzi palea* Cuv. et Val.) larvae using dry and live food. III. Growth of fish and developmental characteristics related to nutrition. *Aquaculture* 48:123-135.

30. Dabrowski KR, Poczyczynski P. (1988a). Laboratory experiment and mass rearing of coregonic fish fed exclusively in dry diets. *Aquaculture* 69:307-316.

31. Dabrowski KR, Poczyczynski P. (1988b). Comparative experiments on starter diets for grass carp and common carp. *Aquaculture* 69:317-332.

32. DeKoven DL. (1990). Evaluation of conventional and purified rations for the Japanese medaka, *Oryzias latipes*, Temminck and Schlegel. MSc Thesis, University of California, Davis.

33. DeKoven DL, Nunez JM, Lester SM, et al. (1992). A purified diet for medaka (*Oryzias latipes*): Refining a fish model for toxicological research. *Lab. Animal Sci.* 42:185-194.

34. Egami N, Etoh H. (1969). Life span data for the small fish, *Oryzias latipes*. *Exp. Geront.* 4:127-129.

35. Egami N, Kyono-Hamaguchi Y, Mitani H, Shima A. (1981). Characteristics of hepatoma produced by treatment with diethylnitrosamine in the fish, *Oryzias latipes*. In: *Phyletic Approaches to Cancer*, Dawe CJ, Harshbarger JC, Kondo S, Sugimura T, Takayama S (eds). Japan Sci Soc Press, Tokyo, pp. 217-226.

36. Fabacher DL, Besser JM, Schmitt CJ, Harshbarger JC, Peterman PH, Lebo JA. (1991). Contaminated sediments from tributaries of the great lakes: chemical characterization and carcinogenic effects in medaka (*Oryzias latipes*). *Arch. Environ. Contam. Toxicol.* 20:17-34.

37. Farber E. (1976). Hyperplastic areas, hyperplastic nodules and hyperbasophilic areas as putative precursor lesions. *Cancer Res.* 36:2532-2533.

38. Fiala S, Fiala AE, Dixon B. (1972). Gamma-glutamyl transpeptidase in transplantable, chemically induced rat hepatomas and 'spontaneous' mouse hepatomas. . *J. Natl. Cancer Inst.* 48:1393-1401.

39. Fujita S, Watanabe T, Kitajima C. (1980). Nutritional quality of *Artemia* from different localities as a living feed for marine fish from the viewpoint of essential fatty acids. In: *The brine shrimp Artemia. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O (eds). Universa Press, Wetteren, Belgium, pp. 277-290.

40. Greenlee W, Poland A. (1978). An improved assay of 7-ethoxycoumarin o-deethylase activity: Induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3 methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Pharmacol. Exp. Ther.* 205:596-605.

41. Halver JE. (1957). Nutrition of salmonid fishes. III. Water-soluble vitamin requirements of chinook salmon. *J. Nutrit.* 62:225-243.

42. Halver JE, Coates JA. (1957). A vitamin test diet for long-term feeding studies. *Prog. Fish. Cult.* 19:112-118.

43. Harris LE. (1984). Effects of a broodfish diet fortified with canathaxanthin on female fecundity and egg color. *Aquaculture* 43:179-183.

44. Hatanaka J, Doke N, Harada T, Aikawa T, Enomoto M. (1982). Usefulness and rapidity of screening for the toxicity and carcinogenicity of chemicals in medaka, *Oryzias latipes*. *Jpn. J. Exp. Med.* 52:243-253.

45. Hawkins WE, Fournie JW, Battalora MSJ, Walker WW. (1991). Carcinoma of the exocrine pancreas in medaka. *J. Aquat. Animal Health* 3:213-220.

46. Hawkins WE, Fournie JW, Overstreet RM, Walker WW. (1986). Intraocular neoplasms induced by methylazoxymethanol acetate in Japanese medaka (*Oryzias latipes*). *J. Natl. Cancer Inst.* 76:453-465.

47. Hawkins WE, Overstreet RM, Fournie JW, Walker WW. (1985a). Development of aquarium fish models for environmental carcinogenesis: tumor induction in seven species. *J. Appl. Toxicol.* 5:261-264.

48. Hawkins WE, Overstreet RM, Walker WW. (1988a). Small fish models for identifying carcinogens in the aqueous environment. *Water Res. Bull.* 24:941-949.

49. Hawkins WE, Overstreet RM, Walker WW. (1988b). Carcinogenicity tests with small fish species. *Aquat. Toxicol.* 11:113-128.

50. Hawkins WE, Overstreet RM, Walker WW, Manning CS. (1985b). Tumor induction in several fish species by classical carcinogens and related compounds. Lewis Publishers, Inc., Chelsea, Michigan, 429-438.

51. Hawkins WE, Walker WW, Overstreet RM, Lytle JS, Lytle TF. (1990). Carcinogenic effects of some polycyclic aromatic hydrocarbons on the Japanese medaka and guppy in waterborne exposures. *Sci. Total Environ.* 94:155-167.

52. Hawkins WE, Walker WW, Overstreet RM, Lytle TF, Lytle JS. (1988c). Dose-related carcinogenic effects of water-borne benzo[*a*]pyrene on livers of two small fish species. *Ecotoxicol. Environ. Safety* 16:219-231.

53. Hendricks JD. (1982). Chemical carcinogenesis in fish. In: *Aquatic Toxicology*, 1, Weber LJ (ed). Raven Press, New York, pp. 149-211.

54. Hickie DE, Dixon DG. (1987). The influence of diet and preexposure on the tolerance of sodium pentachlorophenate by rainbow trout (*Salmo gairdneri*). *Aquat. Toxicol.* 9:343-353.

55. Hinton DE. (1989a). Environmental contamination and cancer in fish. *Mar. Environ. Res.* 28:411-416.

56. Hinton DE. (In Press:a). Structural considerations in teleost hepatocarcinogenesis: gross and microscopic features including architecture, specific cell types and focal lesions. In: *ATLAS of Neoplasms and Related Disorders*, Dawe CJ (ed). Academic Press, New York,

57. Hinton DE. (In Press:b). Toxicologic histopathology of fishes: a systemic approach and overview. In: *Pathobiology of Marine and Estuarine Organisms*, Couch JA, Fournie JF (eds). CRC Press, Boca Raton, FL.

58. Hinton DE, Couch JA. (1984). Pathobiological measures of marine pollution effects. Concepts in Marine Pollution Measurements. Maryland Sea Grant College, University of Maryland, College Park. pp. 7-32.

59. Hinton DE, Baumann PC, Gardner GR, Hawkins WE, Hendricks JD, Murchelano RA, Okihiro MS. (1992) Histopathological Biomarkers. In: Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress. R.J. Huggett, R.A. Kimerle, P.M. Mehrle, Jr., H.L. Bergman (eds.) Lewis Publishers, Inc., Boca Raton, FL. pp. 155-209.

60. Hinton DE, Couch JA, Teh SJ, Courtney LA. (1988b). Cytological changes during progression of neoplasia in selected fish species. *Aquat. Toxicol.* 11:77-112.

61. Hinton DE, Hampton JA, McCuskey PA. (1985). Japanese medaka liver tumor model: review of the literature and new findings. In: *Water Chlorination: Chemistry, environmental impact and health effects*, Chapter 35, Jolley RL, Bull RJ, Davis WP, Katz S, Roberts MH, Jacobs V (eds). Lewis Publishers, Inc., Chelsea, Michigan, pp. 439-450.

62. Hinton DE, Lantz RC, Hampton JA. (1984a). Effect of age and exposure to a carcinogen on the structure of the medaka liver: A morphometric study. *Natl. Cancer Inst. Monogr.* 65:239-249.

63. Hinton DE, Lantz RC, Hampton JA, McCuskey PR, McCuskey RS. (1987). Normal versus abnormal structure: considerations in morphologic responses of teleosts to pollutants. *Environ. Health Perspect.* 71:139-146.

64. Hinton DE, Laurén DJ. (1990a). Liver structural alterations accompanying chronic toxicity in fishes: potential biomarkers of exposure. In: *Biomarkers of Environmental Contamination*, McCarthy JF, Shugart LR (eds). Lewis Publishers, Boca Raton, Florida, pp. 17-57.

65. Hinton DE, Laurén DJ. (1990b). Integrative histopathological approaches to detecting effects of environmental stressors on fishes. *Am. Fish. Soc. Symp.* 8:51-66.

66. Hinton DE, Laurén DJ, Teh SJ, Giam CS. (1988a). Cellular composition and ultrastructure of hepatic neoplasms induced by diethylnitrosamine in *Oryzias latipes*. *Mar. Environ. Res.* 24:307-310.

67. Hinton DE, Teh SJ, Okihiro MS, Cooke JB, Parker LM. (In Press). Phenotypically altered hepatocyte populations in diethylnitrosamine-induced medaka liver carcinogenesis: resistance, growth and fate. *Mar. Environ. Res.* In Press.

68. Hinton DE, Walker ER, Pinkstaff CA, Zuchelkowski EM. (1984b). Morphological survey of teleost organs important in carcinogenesis with attention to fixation. *Natl. Cancer Inst. Monogr.* 65:291-320.

69. Hirshfield MF. (1980). An experimental analysis of reproductive effort and cost in the Japanese medaka, *Oryzias latipes*. *Ecology* 6:282-292.

70. Hoover KL. (1984a). Use of small fish species in carcinogenicity testing. *Natl. Cancer nst. Monogr.* 65:409.

71. Horning WB, Weber CI. (1985). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, pp. 58-75.

72. Ishikawa T, Masakito P, Takayama S. (1984). Usefulness of the medaka, *Oryzias latipes*, as a test animal: DNA repair. *Natl. Cancer Inst. Monogr.* 65:35-43.

73. Ishikawa T, Shimamine T, Takayama S. (1975). Histologic and electron microscopy observations of diethylnitrosamine induced hepatomas in small aquarium fish *Oryzias latipes*. *J. Natl. Cancer Inst.* 55:909-916.

74. Ishikawa T, Takayama S. (1979). Importance of hepatic neoplasms in lower vertebrate animals as a tool in cancer research. *J. Toxicol. Environ. Health* 5:537-550.

75. Jobling M. (1986). Gastrointestinal overload - a problem with formulated feeds? *Aquaculture* 51:257-263.

76. Johns DM, Peters ME, Beck AD. (1980). Nutritional value of geographical and temporal strains of *Artemia*: effects on survival and growth of two species of Brachyuran larvae. In: *International study on Artemia VI. The brine shrimp Artemia. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O et al (eds). Universa Press, Wetteren, Belgium, pp. 290-304.

77. Jones CE. (1984). Animal feed. In: *Official Methods of Analysis of the Association of Official Analytical Chemists. 14*, Williams S (eds). Association of Official Analytical Chemists, Arlington, VA, pp. 152-169.

78. Kaplowitz N. (1980). Physiological significance of glutathione S-transfereases. *Am. J. Physiol. (Gastrointest. Liver Physiol. 2)* 239:6439-6444.

79. Kirchen RV, West WR. (1976). The Japanese medaka. Its care and development, Carolina Biological Supply, Burlington, North Carolina,

80. Klaunig JE, Barut BA, Goldblatt PJ. (1984). Preliminary studies on the usefulness of medaka, *Oryzias latipes*, embryos in carcinogenicity testing. *Natl. Cancer Inst. Monogr.* 65:155-161.

81. Klein-Macphee G, Howell WH, Beck AD. (1980). International study on *Artemia* VII. Nutritional value of five geographical strains of *Artemia* to winter flounder *Pseudopleuronectes americanus* larvae. In: *The brine shrimp Artemia. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O et al (eds). Universa Press, Wetteren, Belgium, pp. 305-312.

82. Klein-Macphee G, Huntting Howell W, Beck AD. (1982). Comparison of a reference strain and four geographical strains of *Artemia* as food for winter flounder (*Pseudopleuronectes americanus*) larvae. *Aquaculture* 29:279-288.

83. Kyono Y. (1978). Temperature effects during and after the diethylnitrosamine treatment on liver tumorigenesis in the fish *Oryzias latipes*. *Eur. J. Cancer* 14:1089-1097.

84. Kyono Y, Egami N. (1977). The effect of temperature during the diethylnitrosamine treatment on liver tumorigenesis in the fish *Oryzias latipes*. *Eur. J. Cancer* 13:1191-1194.

85. Kyono Y, Shima A, Egami N. (1979). Changes in the labeling index and DNA content of liver cells during diethylnitrosamine-induced liver tumorigenesis in *Oryzias latipes*. *J. Natl. Cancer Inst.* 63:71-74.

86. Kyono-Hamaguchi Y. (1984). Effects of temperature and partial hepatectomy on the induction of liver tumors in *Oryzias latipes*. *Natl. Cancer Inst. Monogr.* 65:337-344.

87. Lauff M, Hofer R. (1984). Proteolytic enzymes in fish development and the importance of dietary enzymes. *Aquaculture* 37:335-346.

88. Laurén DJ, Halarnkar PP, Hammock BD, Hinton DE. (1989b). Microsomal and cytosolic epoxide hydrolase and glutathione *S*-transferase activities in the gill, liver, and kidney of the rainbow trout, *Salmo gairdneri*. *Biochem. Pharmacol.* 38:881-887.

89. Laurén DJ, Teh SJ, Hinton DE. (1990). Cytotoxicity phase of diethylnitrosamine-induced hepatic neoplasia in medaka. *Cancer Res.* 50:5504-5514.

90. Leatherland JF, Sonstegard RA. (1982). Bioaccumulation of organochlorines by yearling coho salmon (*Oncorhynchus kisutch* Walbaum) fed diets containing great lakes coho salmon, and the pathophysiological responses of the recipients. *Comp. Biochem. Physiol.* 72C:91-99.

91. Leatherland JF, Sonstegard RA, Holdrinet MV. (1979). Effect of dietary Mirex and PCBs on hepatosomatic index, liver lipid, carcass lipid and PCBs and Mirex accumulation in yearling coho salmon, *Oncorhynchus kisutch*. *Comp. Biochem. Physiol.* 63C:243-246.

92. Lee DJ, Sinnhuber RO, Wales JH et al. (1978). Effect of dietary protein on the response of rainbow trout (*Salmo gairdneri*) to aflatoxin B_1. *J. Natl. Cancer Inst.* 60:317-320.

93. Malins DC, McCain BB, Landahl JT, et al. (1988). Neoplastic and other diseases in fish in relation to toxic chemicals: an overview. *Aquat. Toxicol.* 11:43-67.

94. Maronpot RR, Montgomery Jr CA, Boorman GA, McConnell EE. (1986). National toxicology program nomenclature for hepatoproliferative lesions of rats. *Toxicol. Pathol.* 14:263-273.

95. Marty GD, Nunez JM, Laurén DJ, Hinton DE. (1990a). Age-dependent changes in toxicity of N-nitroso compounds to Japanese medaka (*Oryzias latipes*) embryos. *Aquat. Toxicol.* 17:45-62.

96. Masahito P, Aoki K, Egami N, Ishikawa T, Sugano H. (1989). Life-span studies on spontaneous tumor development in the medaka (*Oryzias latipes*). *Jpn. J. Cancer Res.* 80:1058-1065.

97. Masahito P, Ishikawa T, Sugano H. (1988). Fish tumors and their importance in cancer research. *Jpn. J. Cancer Res.* 79:545-555.

98. Matsushima T, Sugimura T. (1976). Experimental carcinogenesis in small aquarium fishes. *Prog. Exp. Tumor Res.* 20:367-379.

99. McCain BB, Brown DW, Krahn MK, et al. (1988). Marine pollution problems, North American West Coast. *Aquat. Toxicol.* 11:143-162.

100. Mehrle PM, Johnson WW, Mayer Jr FL. (1974). Nutritional effects on chlordane toxicity in rainbow trout. *Bull. Environ. Contam. Toxicol.* 12:513-517.

101. Mehrle PM, Mayer FL, Johnson WW. (1977). Diet quality in fish toxicology: effects on acute and chronic toxicity. *ASTM STP* 634:269-280.

102. Mommsen TP, Walsh PJ. (1988). Vitellogenesis and oocyte assembly. In: *Fish Physiology Vol. XIA*, Hoar WS, Randall DJ (eds). Academic Press, San Diego, pp. 347-406.

103. Mosconi-Bac N. (1987). Hepatic disturbances induced by artificial feed in the sea bass (*Dicentrarchus labrax*) during the first year of life. *Aquaculture* 67:93-99.

104. Nixon JE et al. (1984). Inhibition of aflatoxin B, carcinogenesis in rainbow trout by flavone and indole compounds. *Carcinogen.* 5:615-619.

105. Nunez JM, Hinton DE. (In Prep.). A recirculating system for the culture and maintenance of fish for toxicological research.

106. Ohki H, Aoki K. (1985). Development of visual acuity in the larval medaka, *Oryzias latipes*. *Zool. Sci.* 2:123-126.

107. Olney CE, Schauer PS, McLean S et al. (1980). Comparison of the chlorinated hydrocarbons and heavy metal in five different strains of newly hatched *Artemia* and a laboratory-reared marine fish. In: *International study on Artemia VIII. The brine shrimp Artemia. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O et al (eds). Universa Press, Wetteren, Belgium, pp. 343-352.

108. Pitot HC. (1983). Contributions to our understanding of the natural history of neoplastic development in lower animals to the cause and control of human cancer. *Cancer Surveys* 2:519-537.

109. Pitot HC, Campbell HA, Maronpot R, et al. (1989). Critical parameters in the quantitation of the states of initiation, promotion, and progression in one model of hepatocarcinogenesis in the rat. *Toxicol. Pathol.* 17:594-612.

110. Roberts RJ, Bullock AM. (1989). Nutritional pathology. In: *Fish Nutrition 2nd Edition*, Halver JE (ed). Academic Press, San Diego, pp. 424-473.

111. Rugh R. (1962). Experimental Embryology Techniques and Procedures. Third Edition, Burgess Publishing, Minneapolis, pp. 367.

112. Sachan DS. (1975). Effects of low and high protein diets on the induction of microsomal drug-metabolizing enzymes in rat liver. *J. Nutr.* 105:1631-1639.

113. Schaeffer JL, Tyczkowski JK, Parkhurst CR et al. (1988). Carotenoid composition of serum and egg yolks of hens fed diets varying in carotenoid composition. *Poultry Sci.* 67:608-614.

114. Schauer PS, Johns DM, Olney CE et al. (1980). Lipid level, energy content and fatty acid composition of the cysts and newly hatched nauplii from five geographical strains of *Artemia*. In: *The brine shrimp Artemia. International study on Artemia IX. Vol. 3. Ecology, culturing, use in aquaculture*, Persoone G, Sorgeloos P, Roels O et al (eds). Universa Press, Wetteren, Belgium, pp. 365-373.

115. Schell JD, Cooper KO, Cooper KR. (1987). Hepatic microsomal mixed-function oxidase activity in the Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* 6:717-721.

116. Seidel CR, Johns DM, Schauer PS et al. (1982). Food value of nauplii from reference *Artemia* cysts and four geographical collections of *Artemia* for mud crab larvae. International study on *Artemia* XXVI. *Mar. Ecol. Prog. Ser.* 8:309-312.

117. Sinnhuber RO, Hendricks JD, Wales JH, Putnam GB. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. N.Y. Acad. Sci.* 298:389-408.

118. Sokal RR, Rohlf FJ. (1981). Biometry, 2nd, Freeman Publishing, New York, pp. 733 pages.

119. Stanley RD. (1977). The effect of egg size and size and viability of newly hatched medaka (*Oryzias latipes*) and surf smelt (*Hypomesus pretiosus pretiosus*). MSc Thesis, University of British Columbia.

120. Steven DM. (1949). Studies on animal carotenoid. II. Carotenoid in the reproductive cycle of the brown trout. *J. Exp. Biol.* 26:295-303.

121. Stott WT, Sinnhuber RO. (1987). Dietary protein levels and aflatoxin B_1 metabolism in rainbow trout (*Salmo gairdneri*). *J. Environ. Pathol. Toxicol.* 2:379-388.

122. Tacon AGJ. (1981). Speculative review of possible carotenoid function in fish. *Prog. Fish Cult.* 43:205-208.

123. Takayama S, Ishikawa T. (1977). Comparability of histological alterations during carcinogenesis in animals and man, with special reference to hepatocarcinogenesis in fish. In: *Air pollution and cancer in man*, Mohr et al U (eds). IARC Sci Publ No. 16, Lyon, France, pp. 271-286.

124. Takeuchi K. (1960). The behavior of carotenoid and distribution of xanthophores during development of medaka (*Oryzias latipes*). *Embryol.* 5:170-177.

125. Torrissen OJ. (1984). Pigmentation of salmonids - effect of carotenoid in eggs and start-feeding diet on survival and growth rate. *Aquaculture* 43:185-193.

126. Vogelbein WK, Fournie JW, Van Veld PA, Huggett RJ. (1990). Hepatic neoplasms in the munnichog *Fundulus heteroclitus* from a creosote-contaminated site. *Cancer Res.* 50:5978-5986.

127. Wade AE, White RA, Walton LC et al. (1985). Dietary fat a requirement for induction of mixed-function oxidase activities in starved-refed rats. *Biochem. Pharmacol.* 34:3747-3754.

128. Watanabe T, Itoh A, Murakami A et al. (1984). Effect of nutritional quality of diets given to broodstock on the verge of spawning on reproduction of red sea bream. *Bull. Jap. Soc. Sci. Fish.* 50:1023-1028.

129. Weatherley AH, Gill HS. (1981). Recovery growth following periods of restricted rations and starvation in rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* 18:195-208.

130. Weatherley AH, Gill HS. (1987). The Biology of Fish Growth, Chapter 1, Academic Press, New York,

131. Weibel ER. (1980). Stereological Methods, Vol. II. Academic Press, New York, NY.

132. Wyllie AH, Kerr JFG, Cumi AR. (1980). Cell death: The Significance of apoptosis. *Intl. Rev. Cytol.* 68:251-306.

133. Yamamoto M, Egami N. (1974). Sexual differences and age changes in the fine structure of hepatocytes in the medaka, *Oryzias latipes*. *J. Fac. Sci. Univ. Tokyo IV.* 13:199-210.

134. Yamamoto T. (1963). Hereditary and nonheritable vertebral anchylosis in the medaka, *Oryzias latipes*. *Jap. J. Genet.* 38(1):36-47.

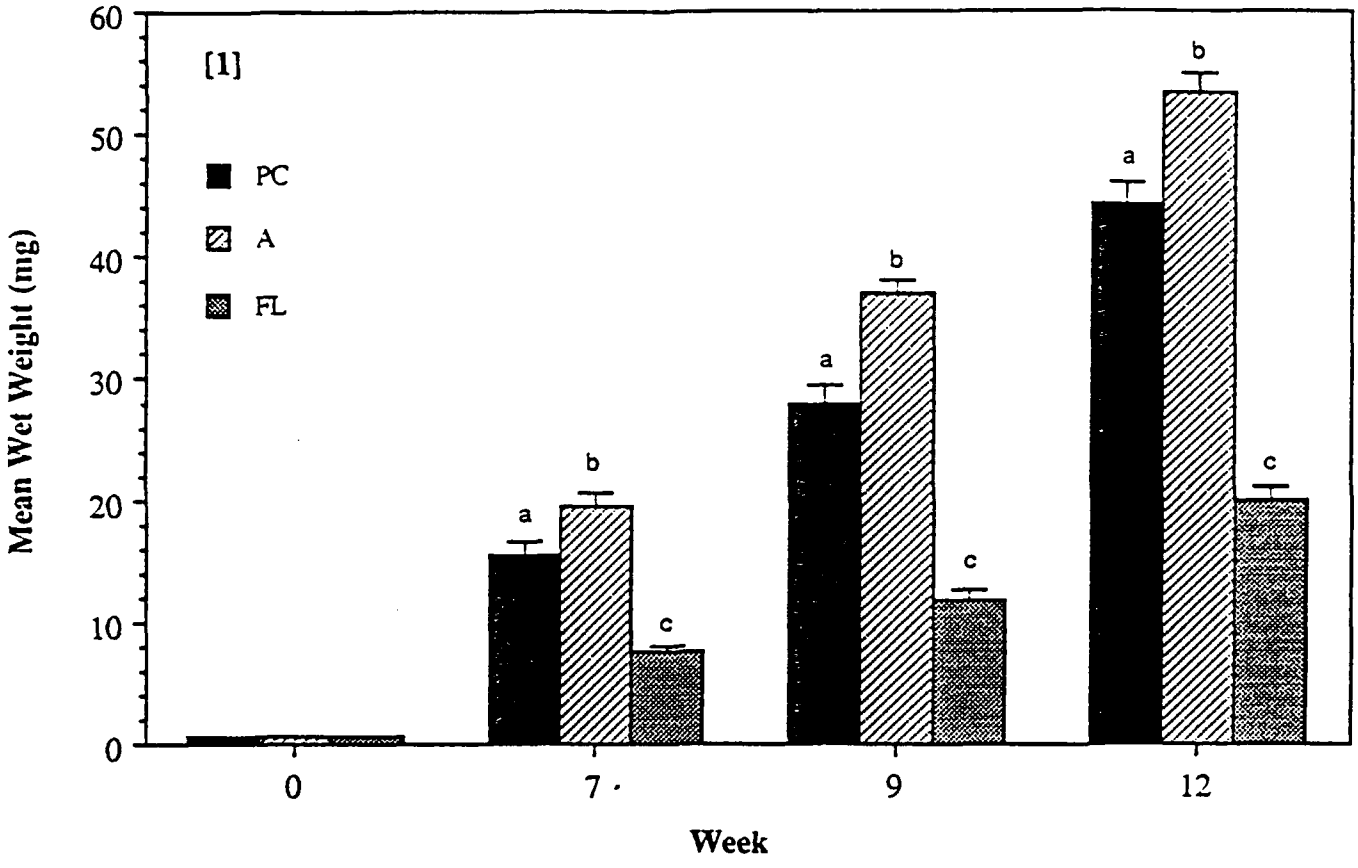
135. Yamamoto T. (1975). Medaka (killifish). Biology and strains. Series of stock culture in biological field, Keigaku Publishing Company, Tokyo,

136. Yu TC, Sinnhuber RO, Hendricks JD. (1979). Reproduction and survival of rainbow trout (*Salmo gairdneri*) fed linolenic acid as the only source of essential fatty acids. *Lipids* 14:572-575.

137. Zongzhu Y, Sato K, Hiroyuki T et al. (1982). Changes in activities of uridine diphosphatglucuronyl-transferases during chemical hepatocarcinogenesis. *Gann* 73:239-248.

FIGURE 1

Trial 1. Average wet weight [1] and average standard length [2] ($\pm$ SE) of medaka reared on Flake (FL), Purified Casein (PC) and live *Artemia* (A) diets. Differences were ranked by the Scheffe test. Bars within same sample period showing different letters (a, b, c) are significantly different $P \leq 0.05$.


[1]
PC
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Mean Wet Weight (mg)
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7
9
12
Week

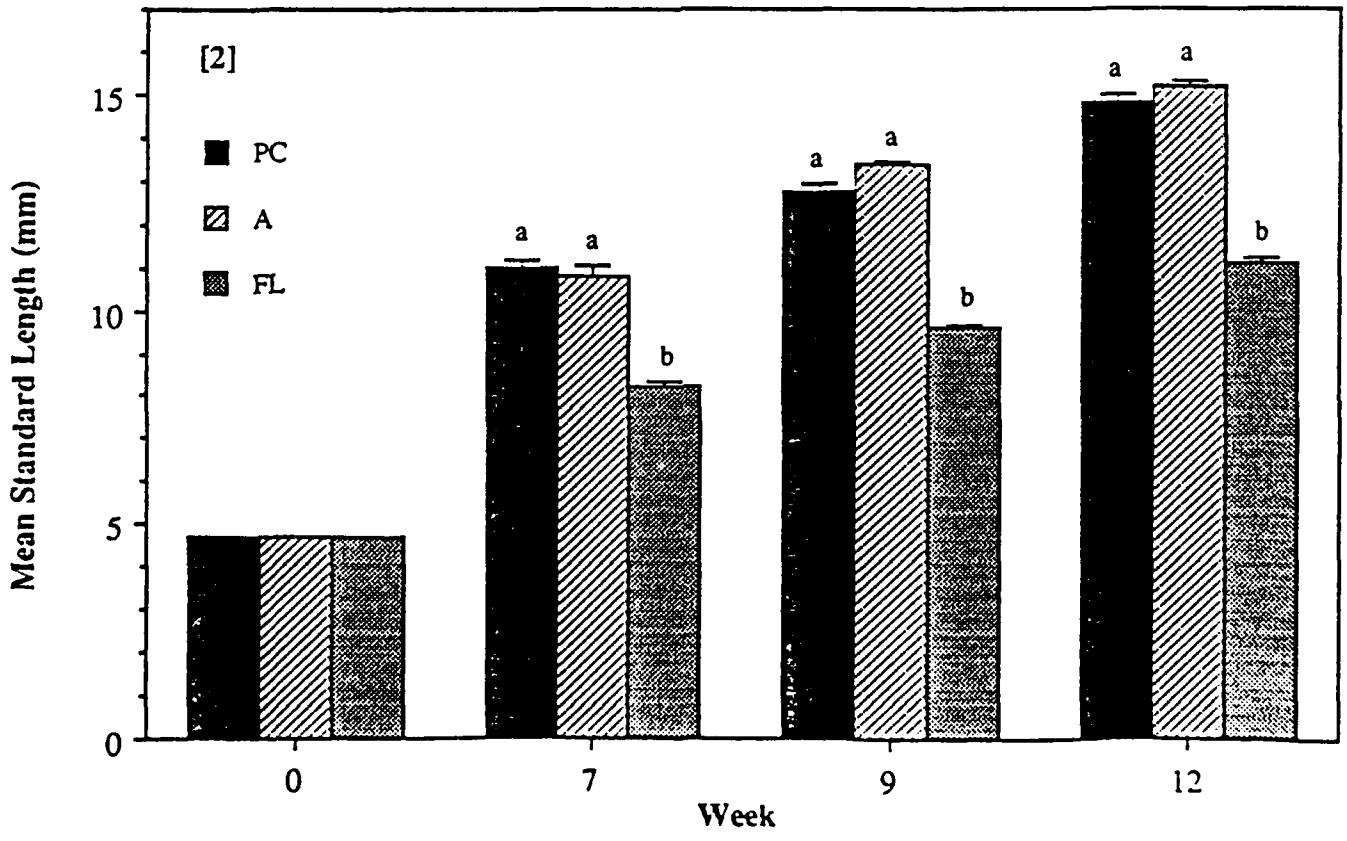

[2]
PC
A
FL
Mean Standard Length (mm)
15
10
5
0
a
a
b
a
a
b
a
a
b
0
7
9
12
Week

FIGURE 2

Trial 2. Effect of diet on individual wet weight (Mean $\pm$ SE) of medaka. Standard errors that are not visible are contained within the symbols.

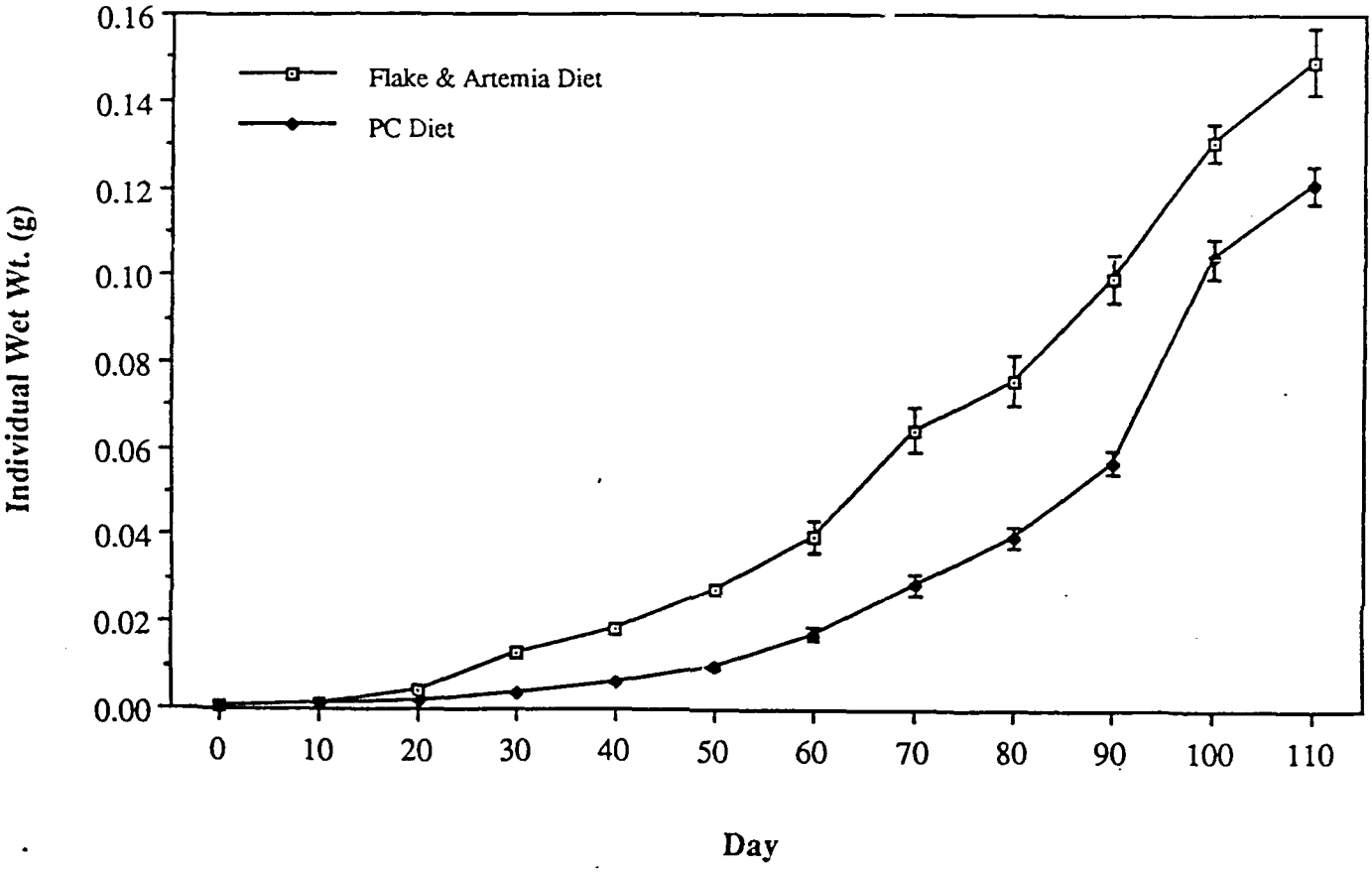

Flake & Artemia Diet
PC Diet
Individual Wet Wt. (g)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
70
80
90
100
110
Day

FIGURE 3

Trial 2. Average instantaneous growth rates (IGR; $\pm$ SE) using wet weight of medaka reared on Purified Casein (PC) and Flake/*Artemia* (F/A) diets. *Denotes IGRs within same sample period that are significantly different ($P \leq 0.05$).

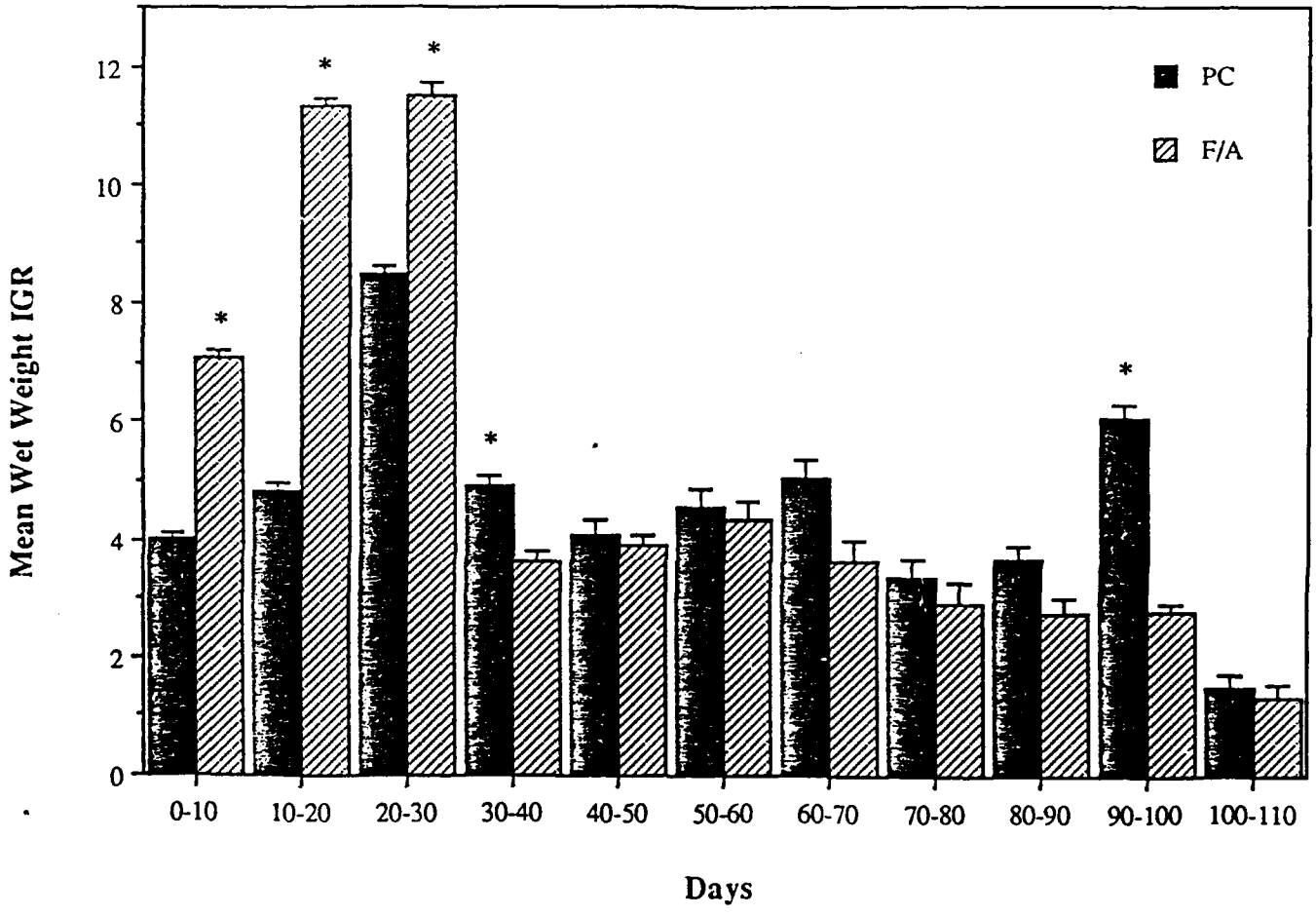
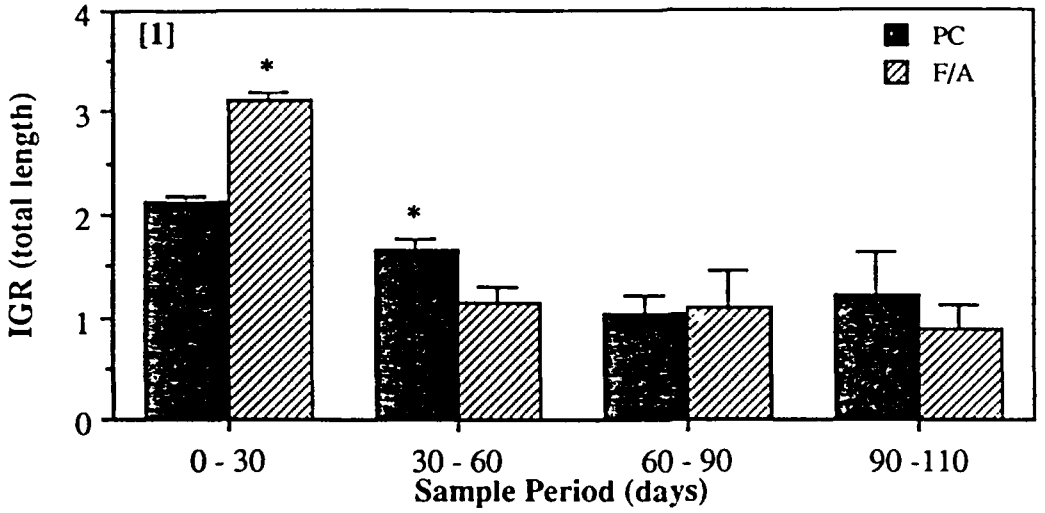
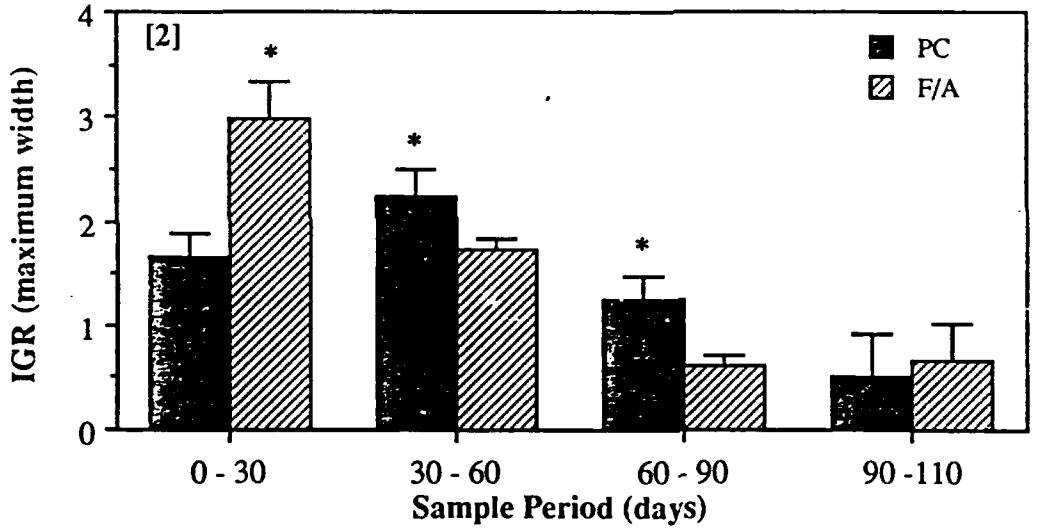

Mean Wet Weight IGR
12
10
8
6
4
2
0
PC
F/A
*
*
*
*
*
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100-110
Days

FIGURE 4

Trial 2. Average instantaneous growth rates (IGR; $\pm$ SE) for total length [1], maximum width [2], and total depth [3] of medaka reared on Purified Casein (PC) and Flake/*Artemia* (F/A) diets. *Denotes IGRs within same sample period that are significantly different ($P \leq 0.05$).


[1]
PC
F/A
IGR (total length)
0
1
2
3
4
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)


[2]
PC
F/A
IGR (maximum width)
0
1
2
3
4
*
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)

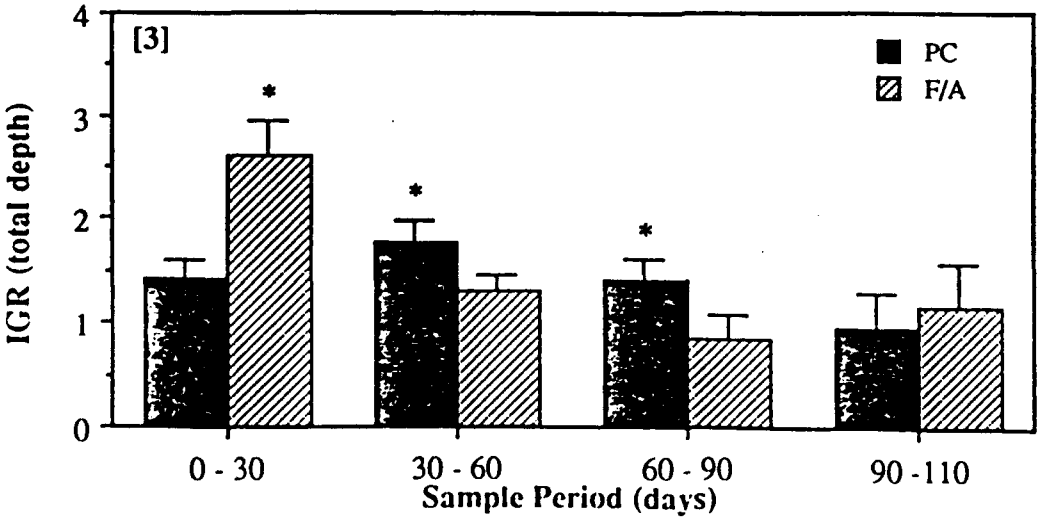

[3]
PC
F/A
IGR (total depth)
0
1
2
3
4
*
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)

FIGURE 5

Normal medaka (Purified Casein, PC-diet) versus medaka with congenital "wavy-tail" and acquired Flake/*Artemia* (F/A) diet-related abnormalities. The fish with the "wavy-tail" was not part of the diet studies. Magnifications of gross photographs, radiographs, and mid-sagittal histologic sections vary slightly, but all three fish are 2 to 3 cm long.

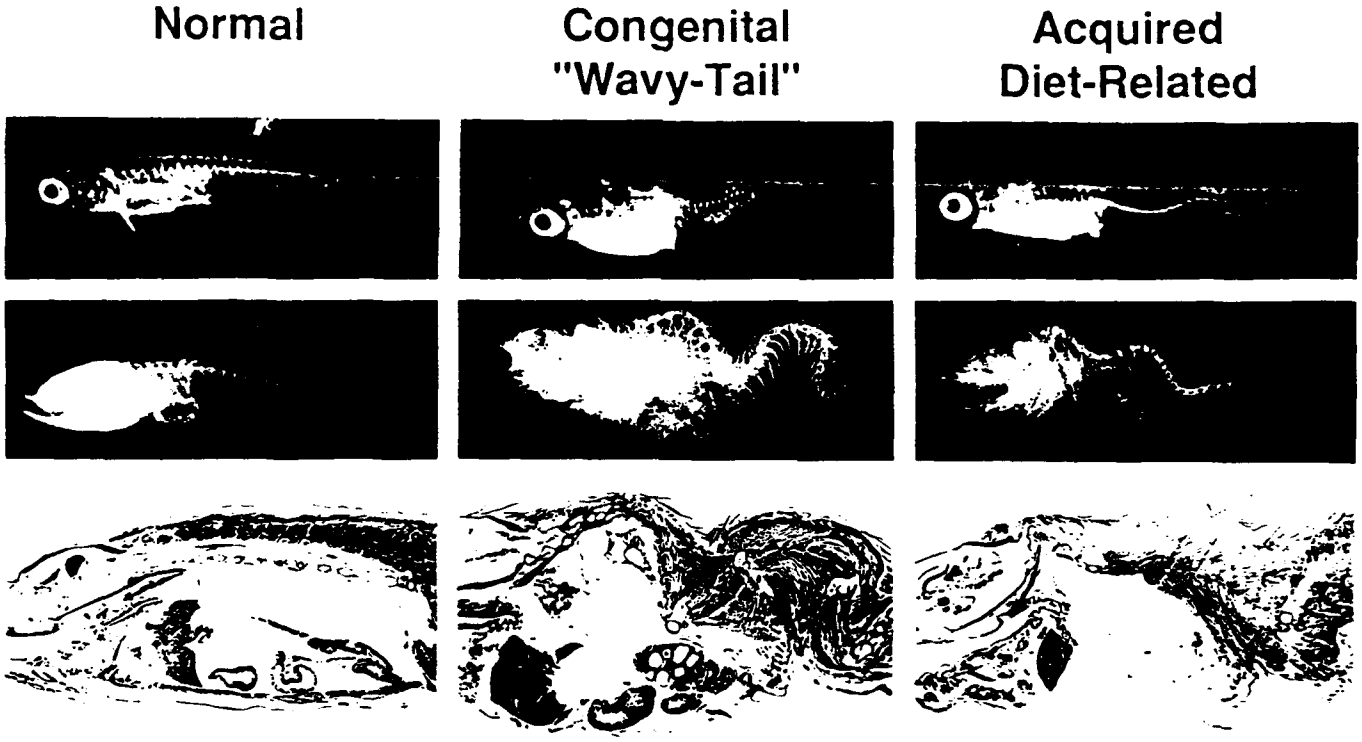

Normal
Congenital
"Wavy-Tail"
Acquired
Diet-Related

FIG. 6 Effect of PC and Flake/Artemia Diet on Wet Weight Increase

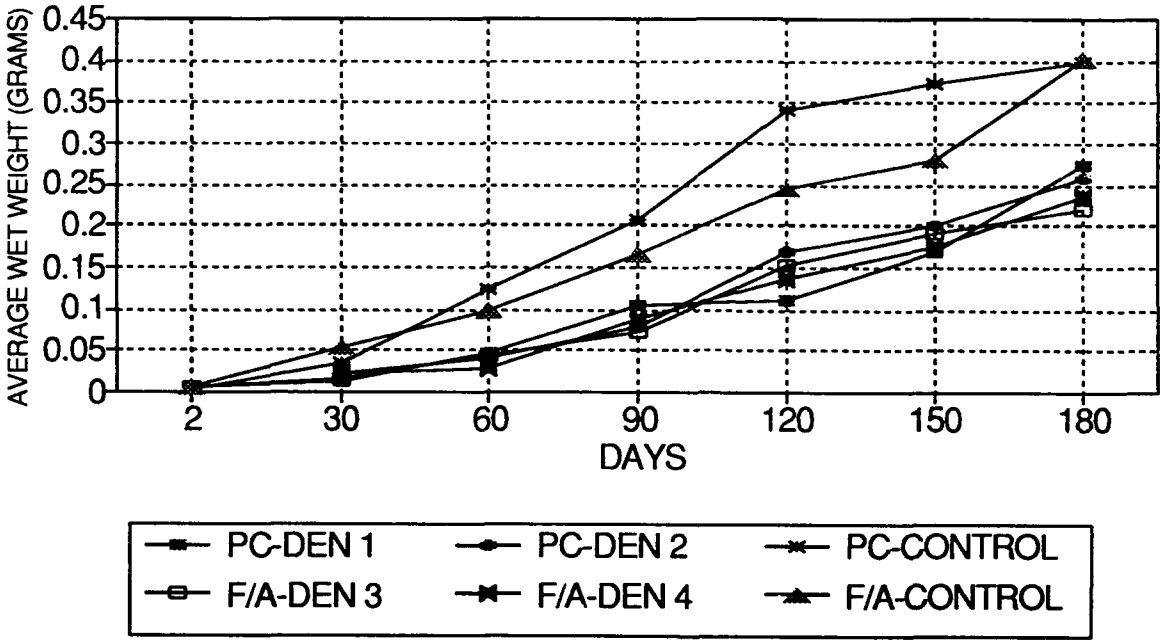
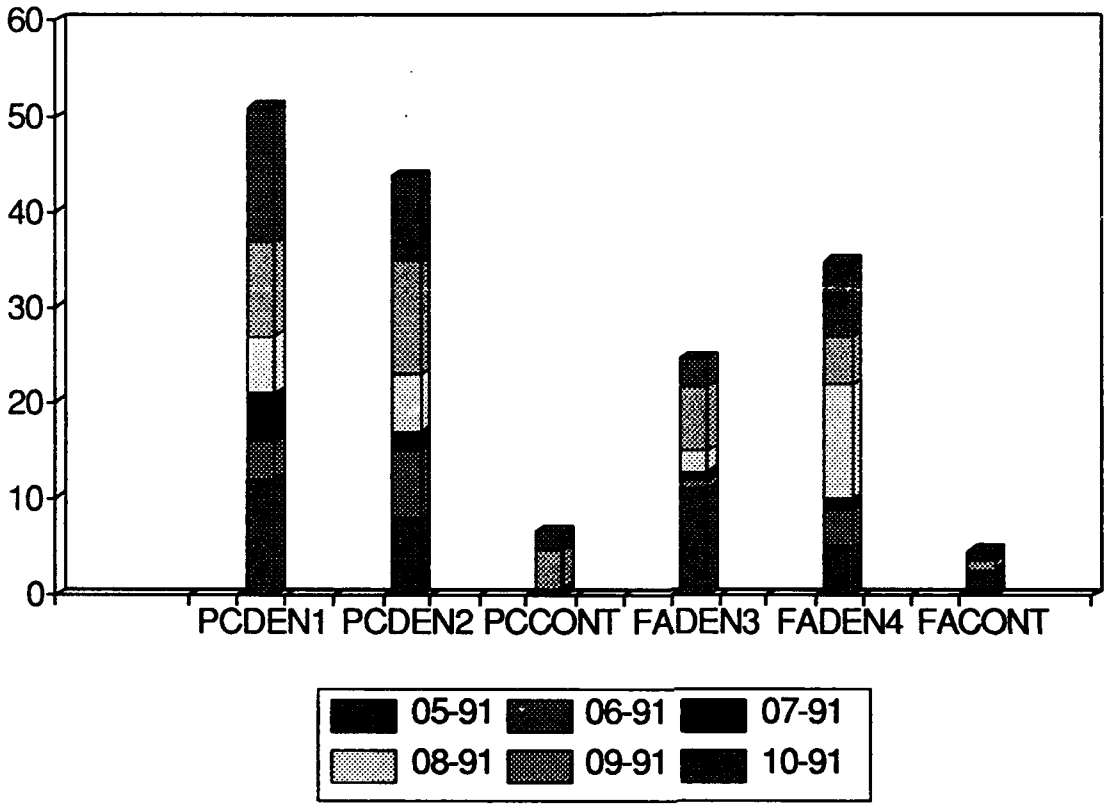


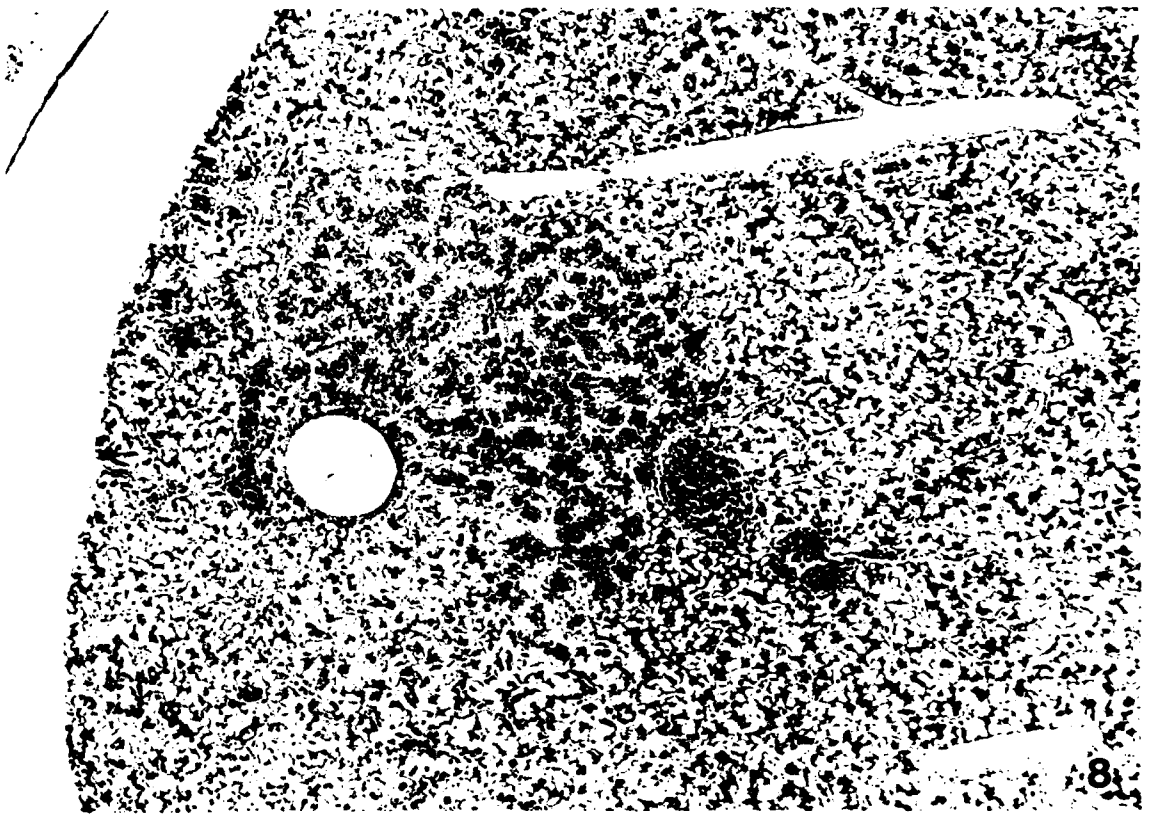
Fig. 7. Mortality in Individual Aquaria



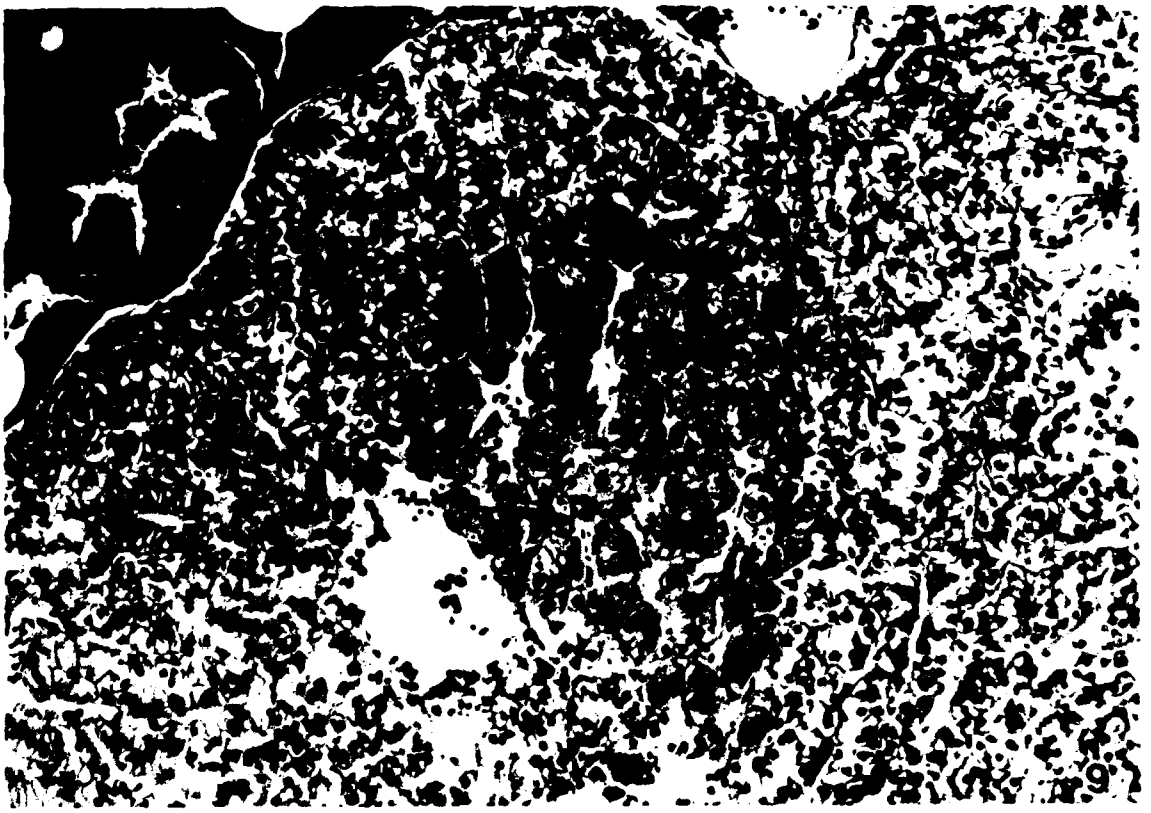
LEGENDS FOR FIGURES

Figure 8. This basophilic focus, of approximate lobular dimension, (325 µm diameter) contains dark-staining basophilic hepatocytes. Note similarity of cellular dimensions in focus and in surrounding hepatocytes. Tubular architectural pattern of hepatic parenchyma is enhanced in this focus. Female medaka fed the F/A diet and exposed for 48 hrs to 350 ppm DEN. Fish was fixed at 6 months after onset of exposure. Hematoxylin and eosin stain X 112.

Figure 9. Eosinophilic focus deep within liver section shows some enlarged hepatocytes and others of near normal dimensions. Component cells of focus illustrate two variants, the granular eosinophilic cells (large arrows) and the eosinophilic cell with glycogen remnants (small arrows). The majority of the cells are hypertrophic and multiple nucleated forms are seen. Female medaka fed the PC diet before and after a 48 hr bath exposure to 350 ppm DEN. Lesion was detected at 6 months after onset of exposure. Hematoxylin and eosin stain X 225.



8



9

LEGENDS FOR FIGURES

Figure 10. Clear cell focus (arrows) at margin of liver section. Cells of foci appear larger than the surrounding cells. Histochemistry in prior companion studies shows glycogen in "clear" areas. Margin is indicated by arrows. This lesion appeared in the liver of a female medaka fed the PC diet. Fish was exposed for 48 hrs to 350 ppm DEN and lesion was detected at 5 months after initiation of exposure. Hematoxylin and eosin stain X 112.

Figure 11. Higher magnification view of the focus illustrated in fig. 10. Cells of focus are larger and stain less than adjacent cells at bottom of field (arrows). Within individual clear cells, observe absence of smooth, rounded margins characteristic of fat vacuoles. A light gray material (C) surrounds clear areas within clear cells. This represents remaining elements in cytoplasm which take up stain. See legend for figure 10 for details of diet and exposure. Hematoxylin and eosin stain X 225.

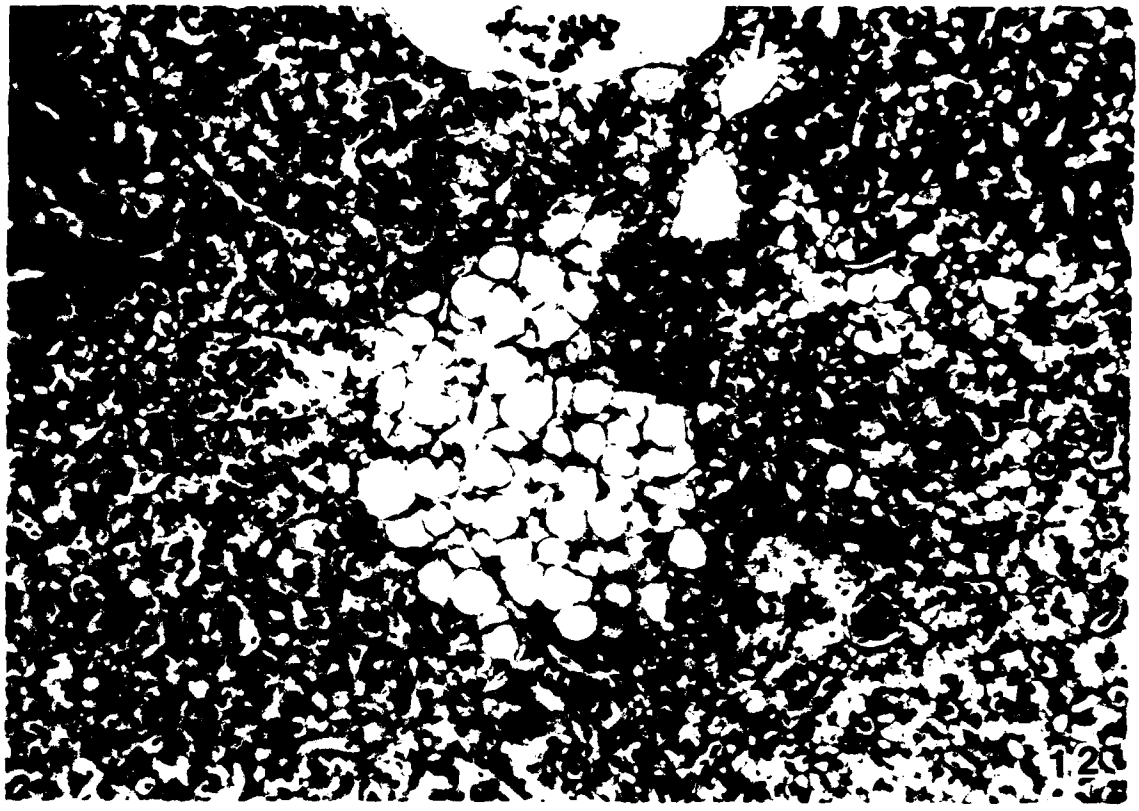

10


C
11

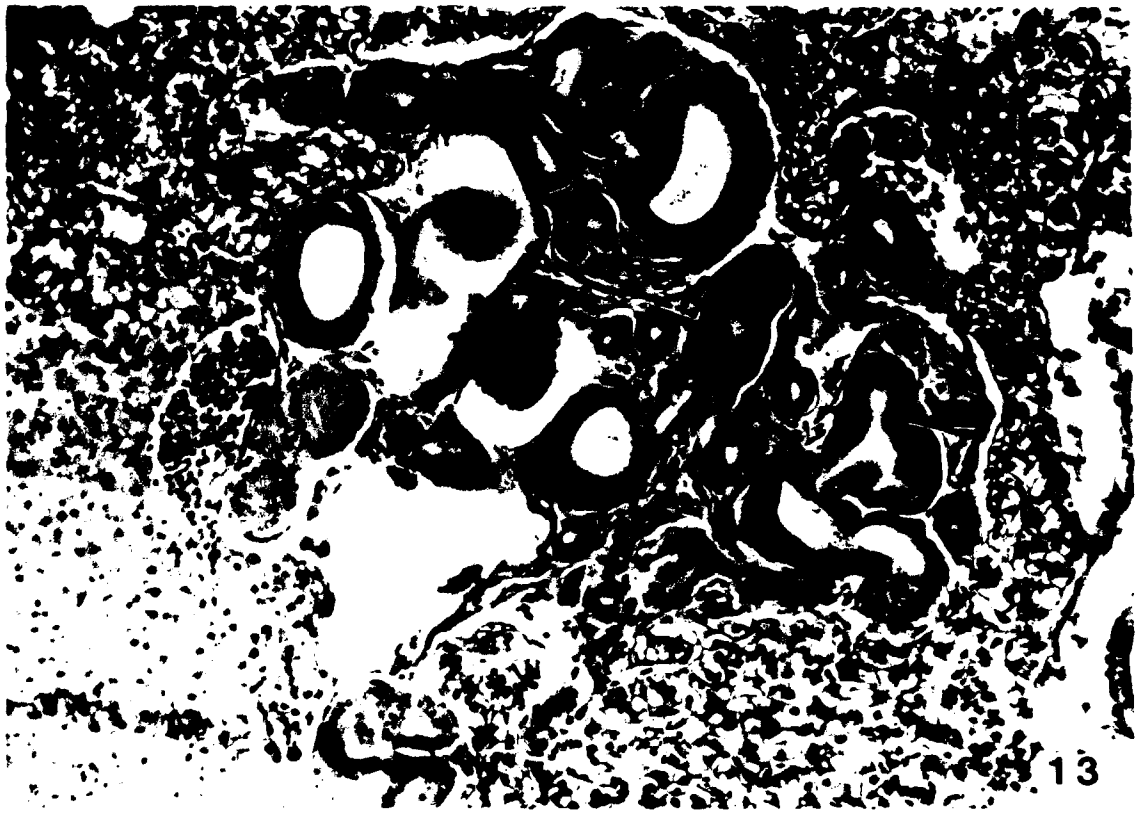
LEGENDS FOR FIGURES

Figure 12. Vacuolated focus reveals large vacuolar profiles with some confluence. Compare with figs. 10 and 11 to observe differences in vacuolated and clear cell foci. Nuclei of cells within this focus show peripheral displacement. This type focus is occasionally encountered in control fish but more often in DEN-treated animals. This lesion was detected at 7 months after onset of exposure (48 hr bath 350 ppm DEN). Fish was a female and was fed the PC diet. Hematoxylin and eosin stain X 225.

Figure 13. Cholangioma involving intrahepatic bile ducts. Columnar to cuboidal epithelial cells continue to form mural elements of biliary passageways, however, "piling up" of nuclei is seen. Early stages of nuclear atypia are indicated by large elongated nuclei (arrows). All epithelial cells continue to appear surrounded by their basal laminae and no invasion of parenchyma is apparent. Male medaka fed the F/A diet and sampled at 6 months after onset of a 48 hr bath exposure to 350 ppm DEN. Hematoxylin and eosin stain X 225.



12

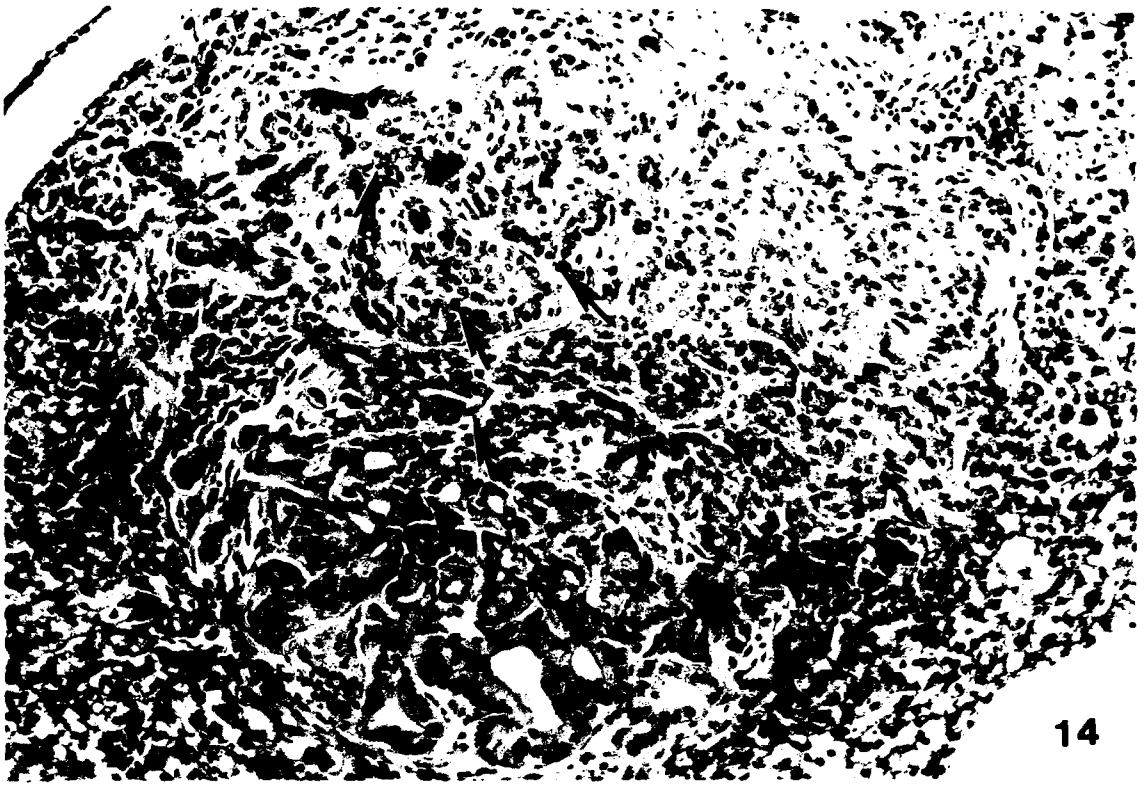


13

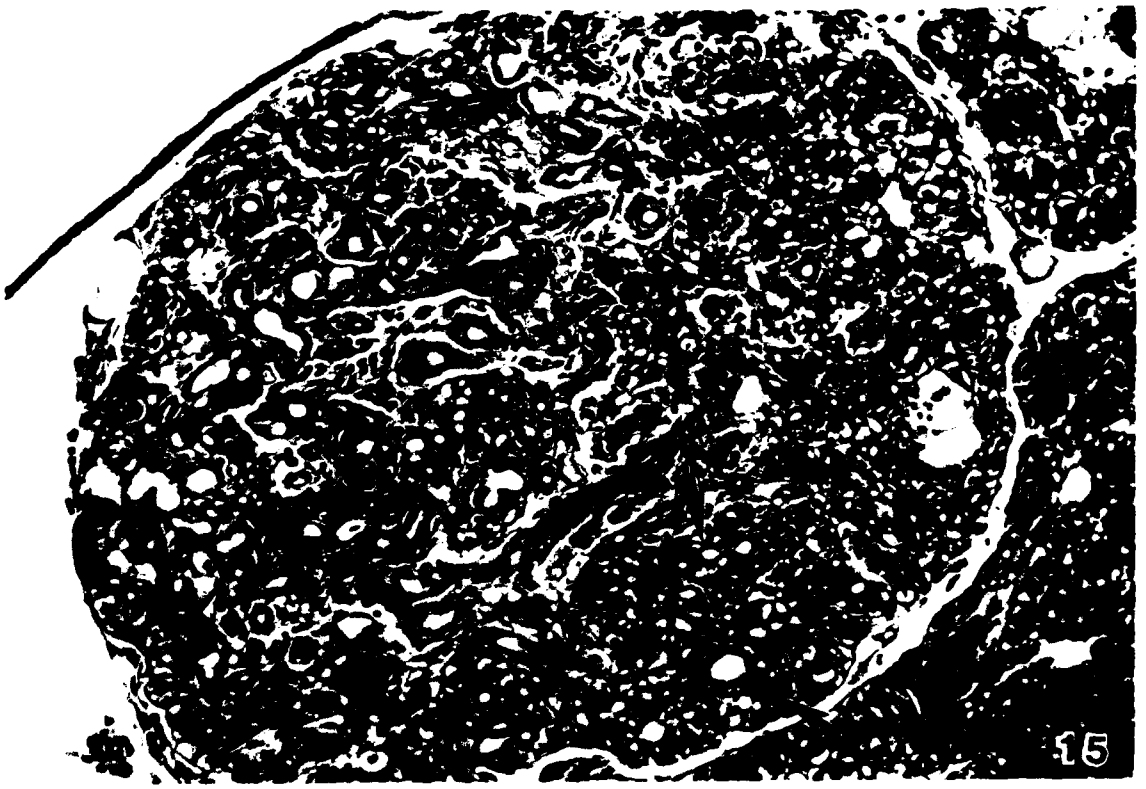
LEGENDS FOR FIGURES

Figure 14. Cholangiocarcinoma. Compare with fig. 13. Cells of carcinoma reveal a much higher incidence of pleomorphic nuclei. Cellular pattern is less like a duct and more solid, trabecular. Note the retention of duct-like structure at bottom of lesion. Cells within middle of iesion have proliferated until they appear as continuous sheets. At top left of field the lesion shows invasion of adjacent hepatic parenchyma. Arrows point to mitotic figures. Male medaka fed F/A diet before and after a 48 hr bath exposure to 350 ppm DEN. Fish was sampled four months after onset of exposure. Hematoxylin and eosin stain X 225.

Figure 15. Mixed hepato- and cholangio- cellular carcinoma. Large, spherical lesion contains both ductular and ductal resembling elements (cholangiocellular component). However, cells at the bottom of lesion and between ductlike structures resemble hepatocytes (arrows). Female medaka fed the PC diet before and after a 48 hr bath exposure to 350 ppm DEN. Fish was sampled at three months after onset of exposure. Hematoxylin and eosin stain X 225.



14



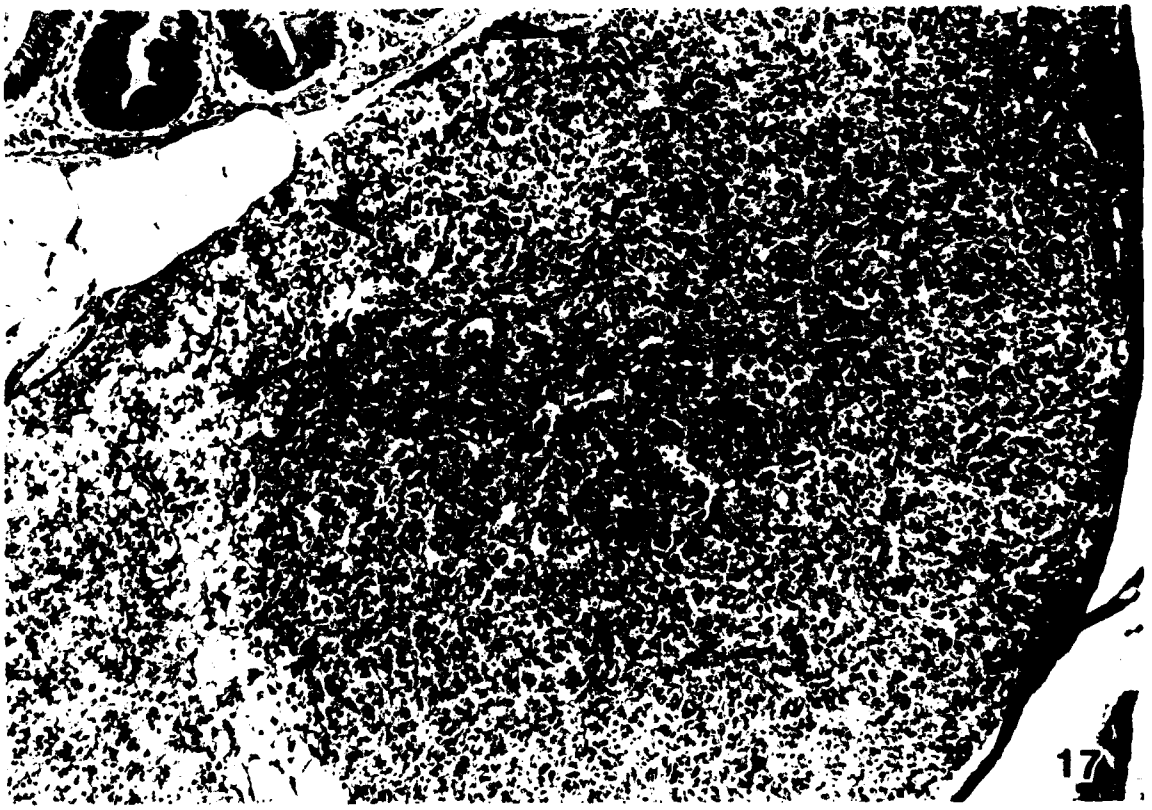
15

LEGENDS FOR FIGURES

Figure 16. Solid, trabecular pattern of hepatocellular carcinoma. Tumor occupies approximately 40% of section area. Note spongiotic lesions at left corner of liver section. Intestine (I) is at top of field and surrounded by fat laden mesentery. Body wall is at bottom and right of field. Male medaka fed F/A diet prior to and after a 48 hr bath exposure to 350 ppm DEN. Sampling and fixation was at 7 months after onset of exposure. Hematoxylin and eosin stain X 45.

Figure 17. Enlarged view of hepatocellular carcinoma in fig. 16. Superficial examination suggests solid sheet of cells. However, finer analysis shows enlarged and hypercellular hepatic tubules forming trabeculae which are compressed together. Extension of the lesion into adjacent liver parenchyma is shown (arrows). Conditions of exposure and time of sampling are given in legend to figure 16. Hematoxylin and eosin stain X 112.

16



17

LEGENDS FOR FIGURES

Figure 18. Extremely large hepatocellular carcinoma occupying majority of liver section shows a central area of necrosis (N) with spongiosis hepatis above and to the right. H = heart; E = Esophagus; P = Pharynx; I = Intestinal bulb. Female medaka sampled at seven months after onset of exposure to a bath of 350 ppm DEN for 48 hrs. Fish was fed F/A diet. Hematoxylin and eosin stain X 45.

Figure 19. Enlarged view of hepatocellular carcinoma shown in fig. 18. Note nuclear pleomorphism in tumor trabeculae. Necrotic areas (arrows) contrast with spongiosis hepatis (SH). Diet, sex, time of sampling and conditions of exposure are in legend to figure 18. Hematoxylin and eosin stain X 112.


E
P
M
H
N
18

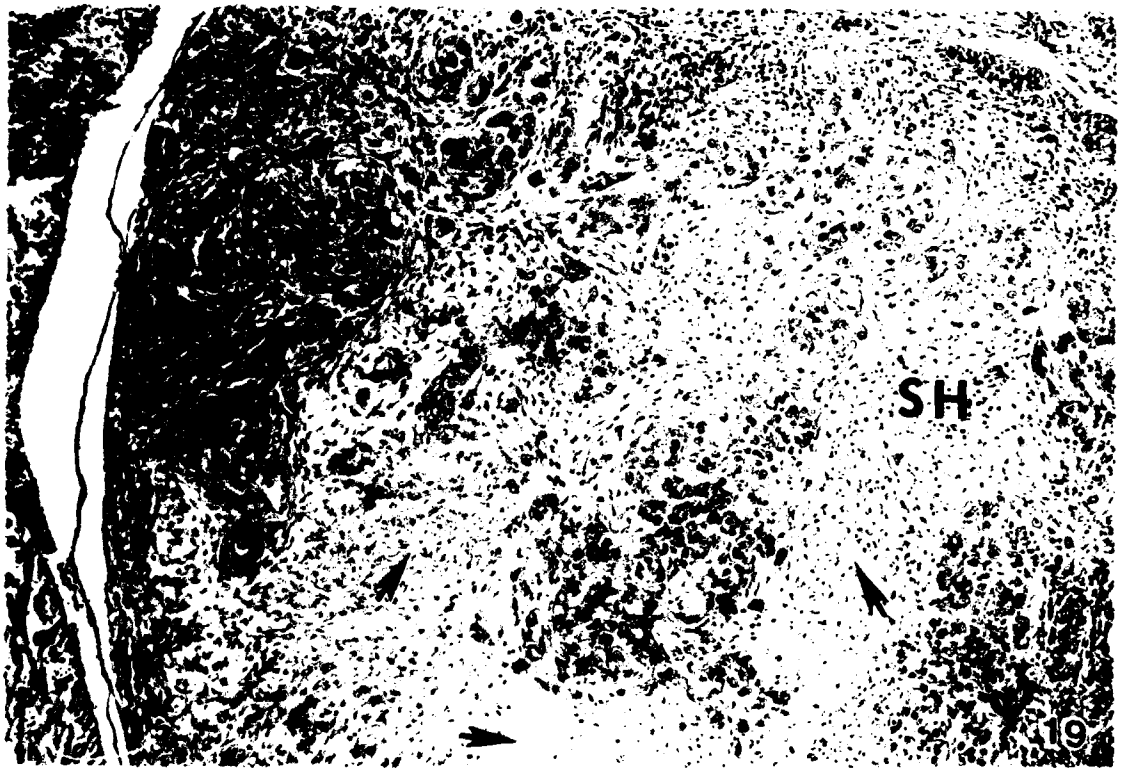

SH
19

Table 1: Composition of Purified Casein (PC) diet for medaka

INGREDIENTS	PERCENT COMPOSITION
Vitamin-free casein	31.0
Wheat gluten	15.0
Dextrin	27.2
Refined soy lecithin	5.2
BML-2 vitamin mix (-C)[a]	4.0
Egg albumin	4.0
BTM mineral mix[b]	3.0
Non-nutritive bulk	3.53
Corn oil	2.0
Cod liver oil	5.0
Tert-butyl hydroquinone	0.002
Vitamin K	0.005
Vitamin A (500,000 IU/G)	0.0018
Vitamin D (400,000 IU/G)	0.00025
Vitamin E[c] (250 IU/G)	0.0075[d]
Vitamin C[e]	0.05

[a] Contains (mg/kg diet in alphacel): thiamin mononitrate (200), riboflavin (320), nicotinic acid (1,040), Ca-d-pantothenate (600), pyridoxine HCL (120), cobalamine (40), folic acid (200), biotin (40), myo-inositol (7,200), p-amino-benzoic acid (120), BHA (40).

[b] Contains (mg/kg diet): calcium carbonate (630), calcium phosphate (22050), citric acid (68.1), cupric citrate · 2½ H_2O (13.8), ferric citrate ·5H_2O (167.4), magnesium oxide (750), manganese citrate (250.5), potassium iodide (0.3), potassium phosphate dibasic (2430), potassium sulfate (2040), sodium chloride (918), sodium phosphate (64.2), zinc citrate · 2H_2O (39.9).

[c] As dl-α-tocopherol acetate.

[d] Vitamin E was increased to 0.03% in Trial 2 in accordance with NRC recommendations (48).

[e] In Trial 2, vitamin C was included in BML-2 Vitamin mix as ascorbic acid (500mg/kg)

Table 2: Trial 2. Proximate analysis of the diets, Flake (FL-diet), Purified Casein (PC-diet) and Artemia (A-diet).

	DIETS			
		FL-diet	PC-diet	A-diet[a]
Dry matter[b]		94.5	94.4	10.3
Crude Protein[c]		50.3	46.4	59.2
Ether Extract[c]	7.8	12.9	19.4	
Ash[c]		11.8	8.2	11.7

[a] Calculated from Watanabe *et al* (50)

[b] Dry matter as % of wet weight

[c] Chemical analysis as % of dry matter

Table 3: Trial 1. Average wet and dry weights (mean ± SE) of fish reared on Flake (FL), Purified Casein (PC) or live Artemia (A) diets at 24 weeks.

	FL-diet	PC-diet	A-diet
Wet Weight (mg)	73.58 ± 1.43*	107.33 ± 1.61	119.83 ± 0.99
Dry weight (mg)	16.98 ± 1.44*	26.81 ± 1.33	30.15 ± 0.77

*Significantly different ($p \leq 0.05$) from other values in the same row.

Table 4: Trial 1. Instantaneous growth rates [a] (mean ± SE; n=3) of medaka fed Flake (FL), Purified Casein (PC) or live Artemia (A) diets; weeks 0-7, 7-9, 9-12.

	WEEK		
	0-7	7-9	9-12
DIET:			
FL	4.88 ± 0.25*	3.45 ± 0.39	2.46 ± 0.40
PC	6.52 ± 0.78	4.01 ± 0.42	2.22 ± 0.60
A	7.03 ± 1.07	4.56 ± 0.40	1.73 ± 0.42

*Significantly different ($p \leq 0.05$) from other values in the same column.

[a] Instantaneous Growth Rate (IGR) = $\frac{LnW_f - LnW_i}{\#\ days} \times 100$

W_f = final wet weight, W_i = initial wet weight

Ln = natural log

Table 5. Trial 2. Morphometric analysis (total length, maximum width, and maximum depth (mean ± SE) at 0, 30, 60, 90, and 110 days in fish fed Purified Casein (PC) or Flake/*Artemia* (F/A) diets.

Sample	Total Length (mm)		Maximum Width (mm)		Maximum Depth (mm)	
day	F/A	PC	F/A	PC	F/A	PC
0	4.7 ± 0.02	4.7 ± 0.02	0.6 ± 0.01	0.6 ± 0.01	0.8 ± 0.009	0.8 ± 0.009
30	12.0 ± 0.02*	8.8 ± 0.02	1.5 ± 0.01*	1.0 ± 0.01	1.6 ± 0.03*	1.3 ± 0.007
60	16.4 ± 0.21*	13.8 ± 0.19	2.6 ± 0.02*	2.0 ± 0.01	3.0 ± 0.03*	2.2 ± 0.01
90	22.5 ± 0.13*	19.5 ± 0.12	3.0 ± 0.02	2.9 ± 0.02	3.8 ± 0.04*	3.3 ± 0.01
110	26.9 ± 0.09*	24 9 ± 0.21	3.3 ± 0.05	3.1 ± 0.02	4.8 ± 0.02*	4.1 ± 0.03

Values of five replicates (10 fish/replicate).

*Significantly different ($p \leq 0.05$) between the same set of morphometric parameters for the sample sample day.

Table 6. Enzyme activities (nmol/min/mg protein of triplicate assays) in pooled livers of medaka fed Flake/*Artemia* (F/A) and Purified Casein (PC) diets.

Age post-hatch (days)	ECOD		GST*	
	F/A	PC	F/A	PC
35	0.019 ± 0.002	0.022 ± 0.001	4.33 ± 0.110	4.64 ± 0.185
70	0.023 ± 0.001	0.019 ± 0.001	7.17 ± 0.358	7.51 ± 0.381
110	0.029 ± 0.002	0.027 ± 0.001	17.45 ± 0.860	28.80 ± 0.704**

* Activities of ethoxycoumarin O-diethylase (ECOD) and glutathione S-transferase (GST) are in the S9 pooled liver fraction from 20 medaka.

**Different from F/A pool by Spearman Rank Correlation test $P \leq 0.5$.

TABLE 7a. INDIVIDUAL WET WEIGHT (GRAMS) OF DIETHYLNITROSAMINE (DEN) EXPOSED MEDAKA FED A PURIFIED CASEIN DIET (PC-DIET)

DATE	5-3-91	6-1-91	7-01-91	7-31-91	8-30-91	9-29-91	10-29-91	11-28-91
DAY	2	30	60	90	120	150	180	210
(DEN 1 = REPLICATE 1)								
1	0.0032	0.0204	0.0423	0.1611	0.0990	0.1490	0.3923	0.2344
2	0.0117	0.0149	0.1432	0.0615	0.0990	0.2440	0.3253	0.4354
3	0.0071	0.0129	0.0231	0.0547	0.1290	0.1959	0.2797	0.3971
4	0.0042	0.0028	0.0291	0.0718	0.2070	0.3077	0.3675	0.3379
5	0.0030	0.0088	0.0824	0.1375	0.0420	0.2682	0.2276	0.4937
6	0.0057	0.0057	0.0330	0.0618	0.1430	0.1109	0.2980	0.2753
7	0.0062	0.0108	0.0841	0.1193	0.1270	0.1234	0.2246	0.3036
8	0.0036	0.0206	0.0202	0.1552	0.0830	0.0890	0.2275	0.1526
9	0.0090	0.0214	0.0091	0.0770	0.1400	0.0654	0.1705	0.2973
10	0.0037	0.0034	0.0053	0.1432	0.0510	0.1179	0.2332	0.8491
AVG	0.0057	0.0122	0.0472	0.1043	0.1120	0.1671	0.2746	0.3776
(DEN 2 = REPLICATE 2)								
1		0.0414	0.0087	0.0265	0.1300	0.2174	0.3272	0.3505
2		0.0098	0.0344	0.1768	0.1950	0.1466	0.1768	0.3201
3		0.0072	0.0720	0.1224	0.1580	0.1054	0.2388	0.1984
4		0.0377	0.0461	0.0542	0.1720	0.3159	0.3148	0.2199
5		0.0095	0.0431	0.0478	0.1420	0.2322	0.1336	0.4021
6		0.0223	0.0370	0.0982	0.1280	0.2612	0.3760	0.2869
7		0.0273	0.0588	0.0643	0.1450	0.0434	0.2714	0.4418
8		0.0041	0.0240	0.0425	0.2720	0.1337	0.3144	0.2248
9		0.0513	0.0239	0.1381	0.0910	0.2836	0.2711	0.2681
10		0.0063	0.0688	0.0362	0.2460	0.2753	0.1670	0.1475
AVG		0.0217	0.0417	0.0807	0.1679	0.2015	0.2591	0.2861
(DEN 5 = CONTROLS)								
1	0.0036	0.0126	0.0531	0.2738	0.4470	0.4904	0.4273	0.3577
2	0.0065	0.0329	0.0824	0.1824	0.2800	0.3289	0.5598	0.4188
3	0.0045	0.0378	0.1420	0.2270	0.2940	0.2680	0.3039	0.5299
4	0.0025	0.0564	0.2132	0.1525	0.3390	0.4024	0.3011	0.3923
AVG	0.0043	0.0349	0.1227	0.2089	0.3400	0.3724	0.3980	0.4247

TABLE 7b. INDIVIDUAL WET WEIGHT (GRAMS) OF DIETHYLNITROSAMINE (DEN) EXPOSED MEDAKA FED A FLAKE & ARTEMIA DIET (F/A-DIET)

DATE	5-3-91	6-1-91	7-01-91	7-31-91	8-30-91	9-29-91	10-29-91	11-28-91
DAY	2	30	60	90	120	150	180	210
(DEN 3 = REPLICATE 1)								
1	0.0038	0.0055	0.0219	0.0332	0.3060	0.3550	0.3417	0.3419
2	0.0112	0.0069	0.0747	0.0242	0.1340	0.2271	0.1969	0.2701
3	0.0071	0.0070	0.0198	0.0768	0.0850	0.2414	0.1944	0.3177
4	0.0083	0.0075	0.0739	0.0881	0.2440	0.2144	0.1468	0.2314
5	0.0195	0.0318	0.0209	0.1141	0.0920	0.1250	0.2722	0.2618
6	0.0059	0.0525	0.0597	0.0332	0.0850	0.1908	0.2148	0.3716
7	0.0036	0.0235	0.0461	0.1310	0.1650	0.2761	0.2776	0.3122
8	0.0016	0.0037	0.0141	0.0183	0.1170	0.0564	0.1388	0.0944
9	0.0031	0.0040	0.0804	0.0656	0.1900	0.1035	0.1342	0.2389
10	0.0015	0.0239	0.0401	0.1575	0.1270	0.1092	0.3018	0.4332
AVG	0.0066	0.0166	0.0452	0.0742	0.1545	0.1899	0.2219	0.2873
(DEN 4 = REPLICATE 2)								
1		0.0011	0.0248	0.1719	0.1470	0.1958	0.2268	0.3405
2		0.0117	0.0395	0.0996	0.2010	0.1641	0.1980	0.2394
3		0.0279	0.0620	0.0586	0.1570	0.2339	0.2057	0.2786
4		0.0234	0.0271	0.0441	0.0880	0.2007	0.2987	0.2265
5		0.0624	0.0255	0.0512	0.2230	0.2293	0.2507	0.1498
6		0.0184	0.0258	0.1302	0.0910	0.1226	0.2126	0.3165
7		0.0100	0.0070	0.1216	0.1170	0.1715	0.2990	0.3277
8		0.0109	0.0074	0.0878	0.1570	0.1012	0.2060	0.2028
9		0.0571	0.0432	0.0475	0.1140	0.1529	0.1600	0.3235
10		0.0053	0.0419	0.0602	0.0830	0.2005	0.2909	0.3176
AVG		0.0228	0.0304	0.0873	0.1378	0.1773	0.2348	0.2723
(DEN 6 = CONTROLS)								
1	0.0096	0.0167	0.0768	0.1865	0.3390	0.3335	0.3794	0.3256
2	0.0050	0.0454	0.0873	0.1452	0.2040	0.2796	0.4528	0.4276
3	0.0069	0.1092	0.1321	0.2614	0.2790	0.2532	0.4123	0.4913
4	0.0035	0.0392	0 0927	0.0740	0.1610	0.2642	0.3710	0.4301
AVG	0.0063	0.0526	0.0972	0.1668	0.2458	0.2826	0.4039	0.4186

Table 8a. Den Exposure Mortality Data

	PC Diet		F/A Diet	
	Exposed	Control	Exposed	Control
4/29/91	0	0	0	0
4/30/91	1	0	1	0
5/l/91	0	0	0	0
PERCENT =	0.19%			

Table 8b DEN Post-Exposure Mortality Data

	PC-Diet			F/A-Diet	
	Exposed DEN #1	Exposed DEN #2	Control DEN #5	Exposed DEN #3	Exposed DEN #4
5/91	12	8	1	11	5
6/91	4	7	0	1	4
7/91	5	2	0	1	1
8/91	6	6	0	2	12
9/91	10	12	4	7	5
10/91	14	9	2	3	8
TOTAL	51	44	9	25	35
PERCENT	95 = 19%		9%	60 = 12%	

Table 9. Classification of liver alterations in hematoxylin and eosin-stained liver sections of medaka exposed to diethylnitrosamine.

FOCI OF CELLULAR ALTERATION	DESCRIPTION
General Features	
Size	Varies from small collection of cells to large lesions occupying 30% of liver sectional area. Often multiple. Occasionally mixed characteristics.
Border	Distinct. Cells not surrounded by capsule. Share architecture of surrounding parenchyma. One hepatic tubule may continue from adjacent (not involved parenchyma into the focus).
Architecture	Same as surrounding parenchyma.
Cytology	Nuclei usually normal but may be enlarged. Cytoplasm usually normal except for tinctorial properties.
Mitotic Figures	Data incomplete. Most foci don't reveal enhanced mitotic figures.
Specific Categories	
1. Basophilic	Normal to small hepatocytes with marked accentuation of cytoplasmic basophilia. Mitotic figures sometimes encountered.
A. Basophilic granular (Baso gr)	Cytoplasm granular and homogeneously basophilic.
B. Basophilic two-toned (Baso ++)	As above, especially around nucleus and at cell periphery. Cytoplasm also contains clear, pale stained areas thought to represent variable content of glycogen.
2. Eosinophilic	Individual hepatocytes vary in size. Some are quite large. Abnormally large nuclei may be present. Eosinophilia predominates over cytoplasm. Typical hepatocytes contain homogeneous pink cytoplasm.

A. Eosinophilic granular (EO grn)	Nuclei are usually non-remarkable. Cytoplasm is granular.
B. Eosinophilic hypertrophic (EO hyp)	Cells are markedly enlarged and may contain enlarged, atypical nuclei. Multiple nuclei may be present.
C. Eosinophilic proteinaceous (EO pro)	Cytoplasm shows ground-glass or hyalinized appearance. Nuclei as above.
3. Clear Cell	Cells of clear cell foci reveal little to no staining. Companion serial sections are positive for glycogen (PAS* method). Clear white nature of cytoplasm distinguishes these from other two foci. Margin of clear space is irregular as opposed to vacuolated cells (below).
A. Clear-basophilic (Cbas)	Cells are predominantly clear with basophilic peripheral cytoplasm.
B. Clear-eosinophilic (C eos)	Cells are predominantly clear with eosinophilic peripheral cytoplasm.
4. Vacuolated	Cytoplasm is vacuolated with normal nuclei. Vacuoles are generally due to lipid extraction during processing.
A. Vacuolated Focus - large (Vac lge)	Large vacuoles, 1-3, per cell.
B. Vacuolated Focus - small (Vac sm)	Small, multiple vacuoles, very numerous in each cell.
5. Mixed	These share features of the above.
A. Mixed foci	Cells may be of more than one of the above types. Predominant type is named first followed by other types (i.e., mixed basophilic and eosinophilic - basophilic predominant.
B. Amphophilic	Neither basophilic or eosinophilic but intermediate between the two and staining is

enhanced.

NEOPLASMS

1. Hepatocellular Adenoma	Cells may stain basophilic, eosinophilic or a mixture. Lesion presents as a distinct nodular mass with well-defined margin. Component cells are arranged as tubules. Number of cells in an individual tubular profile usually increased.
2. Hepatocellular Carcinoma	No clear margin present. Growth extensions into adjacent tissue are usually seen. Gradations from minimal deviation (well-differentiated) to maximal deviation (poorly differentiated) are seen. Cells may appear as sheets. Nuclei are typically altered and often frequent in number.
3. Cholangioma (Biliary Adenoma)	Enlarged and hyperplastic profiles of tubular epithelium. Nuclei show piling up and slight gradation from normal appearance. Lesion is confined within basal lamina of biliary ducts/ductules.
4. Cholangiocarcinoma	Cells are anaplastic. Abundant nuclear and cytoplasmic atypia. Growth by extension into liver parenchyma and/or into intestine or head kidney. Mass may dissect along hepatic veins into pericardium.
5. Other Tumors	
A. Mixed	Carcinomas of both hepatocellular and cholangiocellular components. Both portions show anaplastic features.
B. Spindle Cell	Component cells are attenuated and arranged as strata. Tumor is thought to be of perisinusoidal fat-storing (Ito) cell origin.

*Periodic Acid Schiff's reagent method for glycogen with diastase as a histochemical control

Table 10

Effect of diet on frequency of liver neoplasms in medaka (*Oryzias latipes*) exposed to 350 ppm diethylnitrosamine for 48 hours

Months after Initiation	1	2	3	4	5	6	7
PC Diet[1]	0/20[3]	0/20	4/20	1/20	2/20	1/20	2/20
FL/A Diet[2]	0/20	0/20	2/20	2/20	5/20	3/20	7/20
Control							
PC diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4
FL/A diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4

[1]PC = Purified casein based diet. Fed from day 1 of hatch through day 21 and daily after 48 hour bath exposure.

[2]FL/A = Tetramin flake diet 5 days/wk. and 2 days/wk *Artemia* nauplii. Fed from day 1 of hatch through day 21 and daily after 48 hour bath exposure.

[3] Tumor bearing medaka per number exposed. Neoplasms included adenoma, cholangioma, hepatocellular carcinoma, cholangiocellular carcinoma, mixed hepato- and cholangio-cellular carcinoma, and spindle cell tumor.

Actual mean exposure concentration was 250.8 ppm (PC-diet fed group) and 257.4 ppm (flake *Artemia* fed group). Means included spectrophotometric assays at 12, 24, 36, and 48 hrs. Statistical analysis (Student's T test) revealed no significant differences between exposure concentrations ($P \leq 0.05$).